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(54) Title: METHODS FOR SCREENING FOR ANTIBIOTICS (57) Abstract <p>The present invention provides methods for identification of antibiotic agents which cause the accumulation of ppGpp in bacterial organisms involving a <i>relA</i>-independent pathway. The methods comprise screening assays in which test compounds are brought into contact with <i>relA</i>⁻ test cells and observing the effect such compounds have on ppGpp levels in the test cells. The invention also provides genetically manipulated <i>relA</i>⁻ test cells which contain a reporter gene, the expression of which is sensitive to the level of ppGpp. The invention also encompasses agents identified by the screening assays, and uses of these agents in the treatment of infectious diseases.</p>		

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METHODS FOR SCREENING FOR ANTIBIOTICS

1. INTRODUCTION

The present invention relates to methods for high-throughput screening for compounds with antibiotic activity. Specifically, the invention relates to high-throughput screens which target mechanisms for accumulation of the nucleotide guanosine-3',5'-bis-pyrophosphate ("ppGpp") by *relA*⁺ independent pathways in bacteria. Such targets include ppGpp synthetase II (PSII) and ppGpp degradase (SpoT), both encoded by the *spoT* locus in bacteria. The invention further relates to novel compounds identified using such screening methods.

2. BACKGROUND

Resistance to currently available antibiotics has created a need for new antibiotic agents. In the United States alone, 19,000 hospital patients die each year due to nosocomial (hospital-acquired) bacterial infections (Service, R., 1995, Science 270:724-727). These infections, caused by organisms such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecium* and *Enterococcus faecalis*, have become increasingly resistant to currently approved antibiotics. For example, significant clinical problems include methicillin-resistant strains of *S. aureus*, which are resistant to all current antibiotics except vancomycin (a drug of last resort because of severe side effects), and a vancomycin-resistant strain of *E. faecium* enterococci which is now found world-wide. The occurrence of vancomycin-resistant enterococci isolated from nosocomial infections rose from 0.4% to 13.6% in the relatively short time span from 1989 to 1993 (Tenover, F.C. and Hughes, J.M., 1996, JAMA 275(4):300-304) (reporting statistics from the Centers for Disease Control and Prevention). Even community-acquired organisms such as *Streptococcus pneumoniae* are increasingly resistant to antimicrobial agents, with a

significant number of isolates being resistant to penicillin and extended-spectrum cephalosporins. *Id.*

The emergence and spread of resistant bacterial organisms are primarily caused by acquisition of drug resistance genes, resulting in a broad spectrum of antibiotic resistance (e.g., extended-spectrum cephalosporin-resistant mutant β -lactamases found in several bacterial organisms). Genetic exchange of multiple-resistance genes, by transformation, transduction and conjugation,; combined with selective pressures in settings such as hospitals where there is heavy use of antibiotic therapies, enhance the survival and proliferation of antimicrobial agent-resistant bacterial strains occurring by, e.g., spontaneous mutants. *Id.* Although the extent to which bacteria develop resistance to antimicrobial drugs and the speed with which they do so vary with different types of drugs, resistance has inevitably developed to all antimicrobial agents (Gold and Moellering, Jr., 1996, New Eng. J. Med., 335(19):1445-1453).

To prevent or delay the buildup of a resistant pathogen population, different chemicals that are effective against a particular disease-causing bacterium must be available. Thus, there is a need to develop methods for identifying compounds which can penetrate and specifically kill the pathogenic bacterial cell, or arrest its growth without also adversely affecting its human, animal, or plant host.

2.1. CURRENT SCREENING METHODS

Because resistance to antibiotics is assuming even greater clinical importance, there is a pressing need to develop more effective methods for antibiotic drug discovery. Traditional approaches to screening for antimicrobial agents include chemical modification of existing drugs and mass screening of compounds for bacterial growth inhibition. The first approach, chemical modification of existing antibiotics, attempts to circumvent bacterial resistance while finding more potent activities. This approach has

shown some success, however, it does not produce new classes of drugs and is unlikely to identify new bacterial processes as targets for drug intervention. The second is to directly test compounds for their ability to inhibit bacterial growth using standard microbiological methods, such as growth inhibition assays where libraries of natural products, semisynthetic, or synthetic chemicals are screened for their ability to kill or arrest growth of the target pathogen or a related nonpathogenic model organism. These tests are useful in that they are fast, uncomplicated, relatively inexpensive and allow for rapid testing of large libraries of compounds. However, such screens are blind to the compound's mechanism of action so that rate of efficacy, selectivity, and resistance remain elusive. That is, the promising lead compounds that emerge from such screens must not only be tested for possible toxicity to the human, animal, or plant host, they also undergo detailed mechanism-of-action studies conducted to identify the affected molecular target and precisely how the drug interacts with this target.

Another approach involves screening for compounds which target the resistance mechanism of known antibiotics. The compounds are then administered in conjunction with known antimicrobial agents. This technique is currently being tested for treating organisms resistant to tetracycline compounds. Tetracycline resistant organisms do not accumulate tetracycline within the cell and actually excrete the drug by cellular efflux pumps. Compounds which are tetracycline analogs that tightly bind to the pumps are given in conjunction with tetracycline to assist tetracycline in reaching its target in the bacterial cell (Service, 1995, Science 270:724-727), *supra*. This approach, however, is complicated and does not affect the underlying resistance mechanism.

2.2. DECIPHERING MECHANISMS OF ANTIBIOTIC ACTION

Once antibiotics are identified, a number of studies can be performed to determine their mechanism of

action and their selectivity. Such analyses can sometimes provide new understanding of basic cellular mechanisms.

For example, sulfonamides (or sulfa drugs), the first important antimicrobial agents identified, are actually
5 antimetabolites and not antibiotics. Sulfanilamide, one of the sulfonamide class drugs, is a structural analog of para-aminobenzoic acid ("PABA"). The mode of action of sulfanilamide was unknown until it was discovered that PABA is required for the synthesis of the essential vitamin, folic
10 acid. Folic acid synthesis is required for bacterial growth since bacteria are not capable of folic acid uptake. Sulfonamides inhibit the bacterial synthesis of folic acid by acting as competitive inhibitors of PABA. For humans, folic acid is also an essential vitamin, but unlike bacteria,
15 humans are capable of uptake of folic acid and can obtain the vitamin through diet. As a result, bacteria, but not humans, are vulnerable to sulfa drugs which inhibit folic acid synthesis. In the sulfa class alone, thousands of chemically modified derivatives have been studied with about 25 of them
20 still in use.

Similarly, much has been learned about peptidoglycan synthesis since the discovery of the penicillin and cephalosporins (peptidoglycan is the critical component in maintaining the shape and rigidity of both Gram positive
25 and Gram negative bacterial organisms). Therefore the discovery of new classes of drugs can broaden the general understanding of bacterial physiology as well as provide for new antibacterial chemotherapeutics.

30 2.3. THE USE OF ANTIBIOTICS TO STUDY THE METABOLISM OF ppGpp

Guanosine-3',5'-bis-pyrophosphate or guanosine tetraphosphate (ppGpp) is a nucleotide which inhibits bacterial growth when it accumulates intracellularly. In the
35 enteric bacterium *Escherichia coli*, there are two enzymes which catalyze the synthesis of ppGpp. One of the enzymes is ppGpp synthetase I (PSI) and is encoded by the *relA* locus.

The PSI enzyme is activated during amino acid starvation which results in what is known as the stringent response (Cashel, 1969, J. Biol. Chem. 244:3133-3141; Cashel and Gallant, 1969, Nature 221:838-841). Although the stringent response was first characterized as a response to amino acid starvation, it is now recognized that ppGpp levels change in response to a variety of stress conditions, including carbon, nitrogen or phosphate starvation, heat shock, osmotic shock and pH changes (Murray and Bremer, 1996, J. Mol. Biol. 259:41-57; Cashel et al., 1996, Escherichia coli and Salmonella Cellular and Molecular Biology, ed. Neidhardt, F.C. ASM Press, Washington, D.C. pp. 1458-1496). The second ppGpp synthetic enzyme is ppGpp synthetase II (PSII), and is encoded by the *spoT* locus.

Certain compounds, including several antibiotics, have been used as tools to study the metabolism of ppGpp in bacteria. For example, Cortay & Cozzzone (Cortay & Cozzzone, 1983, Biochimica et Biophysica Acta, 755:467-473) used polymyxin B and gramicidin, antibiotics which disrupt the cell membrane and thereby exert an antibacterial effect, to study ppGpp since a secondary effect of these antibiotics is a decrease in the rate of ppGpp degradation. The assays used by Cortay and Cozzzone to study the metabolism of ppGpp involved measuring the intracellular levels of nucleotides following drug treatment or amino-acid starvation of bacteria. As a general screening method, such an approach would be time consuming and better suited to studying the metabolic effect of individual agents.

The effects of various antibiotics which interfere with protein synthesis were also investigated for effects on ppGpp degradation. Tetracycline, chlortetracycline and thiostrepton were shown to strongly inhibit ppGpp degradation *in vitro*, and levallorphan (a morphine analogue) moderately inhibited ppGpp degradation *in vitro* (Richter, 1980, Arch. Microbiol. 124:225-332).

However, many of these results obtained *in vitro* have little bearing *in vivo* where direct contact of the

degradase with the unmetabolized antibiotic may not occur. In the case of tetracycline, the inhibition was ascribed to chelation of manganese at high concentrations of the antibiotic. The concentrations required would not be

5 achievable *in vivo*.

An antibiotic produced by *Xenorhabdus luminescens*, 3,5-dihydroxy-4-ethyl-trans-stilbene ("ES"), is thought to inhibit bacterial cell growth via an increase in ppGpp concentration by the *relA*⁺-dependent mechanism (Sundar &

10 Chang, 1992, Antimicrobial Agents and Chemotherapy

36(12):2645-2651). Sundar & Chang reported that ES inhibited growth of a stringent *E. coli*s (*relA*⁺), but did not inhibit growth of an isogenic relaxed strain (*relA*⁻). In addition,

ES caused ppGpp to accumulate in a *relA*⁺ strain but not in
15 *relA*⁻ strain. Furthermore, ES did not appreciably interfere with ppGpp degradase. Together these properties strongly suggested that ES action is to induce *relA*-dependent ppGpp synthesis, which leads to inhibition of growth.

20

3. SUMMARY OF THE INVENTION

The invention relates to high-throughput screens which target ppGpp accumulation in bacterial organisms by *relA*⁺ (PSI) independent pathways. Such targets include, for example and not by way of limitation, ppGpp synthetase II
25 (PSII) and 3'-pyrophosphohydrolase, ("ppGpp degradase") both of which are encoded by the *spoT* locus. The ppGpp degradase enzyme is also referred to as the *spoT* enzyme, "ppGpp hydrolase", and "ppGppase" in the literature.

The invention is based, in part, on applicants' recognition that compounds which cause ppGpp accumulation in
30 a *relA*⁺ (PSI) independent manner, such as by enhancing PSII activity or inhibiting ppGpp degradase, have the potential to be therapeutically beneficial as an antibiotic. This antibiotic property exists since increasing levels of ppGpp
35 inhibit many cellular processes, including ribosomal RNA synthesis, and thus, ribosomes, which are essential for bacterial survival and growth. Deletion of the *spoT* gene,

removes both PSII and ppGpp degradase and is not tolerated in the presence of a wildtype *relA* allele (Sarubbi et al., 1988, Mol. Gen. Genet., 213:214-222), suggesting that accumulation of ppGpp is lethal. Apparently, an adequate level of

5 degradase in the organism is essential to prevent

accumulation of toxic levels of ppGpp. Compounds which, for example, reduce, or inhibit this level of degradase activity are attractive candidates for therapeutic use. Similarly, compounds which enhance PSII activity are also desired.

10 Accordingly, the invention provides methods for screening for potential antibiotic drugs by isolating compounds that, for example, enhance PSII ppGpp production or inhibit ppGpp degradase at the *spoT* locus in bacteria.

The screens of the invention use a bacterial strain
15 lacking PSI activity due to a deletions or mutation in the *relA* gene (also known as "relaxed" strain) and having a wildtype *spoT* gene thus producing normal PSII activity and normal ppGpp degradase activity (PSII synthesizes ppGpp, but under different conditions than PSI). Alternatively, the
20 screen could be performed in strains that have functional or wildtype *RelA* activity, but this activity is controlled and limited to levels that are acceptable and that allow accurate detection of ppGpp accumulation in the presence of active compounds.

25 The invention further comprises assays which utilize recombinant test cells which comprise a reporter gene under the control of a promoter which is either positively or negatively controlled by ppGpp. Chemicals tested which score positive for ppGpp accumulation and growth inhibition in this
30 strain background will be independent of PSI, and thus unique from those previously isolated, such as ES (Sundar and Chang, 1992).

Bacterial strains without PSI activity (e.g., *relA* mutated strains) are advantageously used in the screens of
35 the invention since this eliminates the potential for isolating compounds which causes ppGpp synthesis by the PSI enzyme and allows for the identification of compounds that

specifically affect PSI-independent factors in ppGpp accumulation. This is significant since bacteria which lose the PSI gene by mutation are not uncommon and occur readily among laboratory isolates of *E. coli*. Elimination of PSI
5 dependent synthesis, using *relA* mutant strains, allows for specific targeting of ppGpp degradase and/or PSII synthase.

Strains of *E. coli* with deletions in both the *relA* gene and the *spoT* gene have been obtained in the laboratory. These mutants have no functional ppGpp synthetase activity
10 and do not have detectable intracellular ppGpp. These mutants are further characterized by slow growth, failure to divide normally and multiple amino acid auxotrophies (Xiao et al., 1991, J. Biol. Chem 266:5980-5990). They are rapidly overgrown in mixed cultures with wildtype *E. coli* (V. J.
15 Hernandez, personal communication) These observations suggest that the ppGpp signaling pathways provide some selective advantage to the bacterium and the genes encoding the balanced activities of synthetase and degradase are not readily deleted.

20 The above embodiment is illustrated by way of working examples which provide screens for compounds which either stimulate PSII or inhibit degradase, using a bacterial strain with a defective PSI gene, and growing the strain on a medium which is typically unsuitable for growth of the strain
25 except in the presence of a compound that causes an increase in ppGpp levels.

In another embodiment, ppGpp accumulation caused by compounds which, for example, inhibit ppGpp degradase or, alternatively, enhance PSII activity, are screened using a
30 bacterial strain with a defective PSI gene, which contains a reporter gene responsive to changes in the intracellular concentration of ppGpp.

In a less preferred embodiment, the assay strain has a functional *relA* gene.

35 A biochemical assay may be used to verify that the compounds which provide positive results in the physioclogical assays described above increase intracellular

levels of ppGpp. Compounds which score positive in the first assay can be added to growing cells which are labeled with ^{32}P or ^{33}P orthophosphate. A change in intracellular levels of ppGpp is then assayed by extracting and separating labeled nucleotides, including ATP, GTP, ppGpp and pppGpp e.g., using thin layer chromatography (TLC) and visualization by autoradiography. Since this additional assay allows accurate detection of increases in intracellular levels of ppGpp, it will establish whether promising compounds are true modulators of intracellular ppGpp levels. The separation techniques which can be used are well known in the art. For example, one useful TLC method is described in M. Cashel, 1994, Methods in Molecular Genetics, vol. 3, Molecular Microbiology Techniques, Part A, ed. Adolph, K.W. (Academic Press, New York) pp. 341-356) and is incorporated by reference in its entirety.

Similarly, labeling and visualization methods could be used, as are well known in the art, such as radioactive or other isotopes incorporated into orthophosphate, guanosine or other precursors of ppGpp, combined with autoradiography, mass-spectrometry or other appropriate methods of detection.

In another embodiment, purified or partially purified SpoT protein can be used in an enzymatic assay to confirm that compounds identified in the primary assay inhibit the degradation of ppGpp. Purified or partially purified SpoT is combined with ppGpp or pppGpp in the presence and absence of test compounds. Degradase activity is measured by formation of the products pyrophosphate and GTP or GDP. These products can be detected by quantitation of radioactivity if the substrates are radiolabeled, by quantitation of pyrophosphate or by other methods known in the art.

In another embodiment, the invention relates to novel compounds identified by using such screening methods, as well as the uses of these compounds for developing antibiotic drugs for treatment of infectious diseases in animals.

The benefits of the approach taken in the present invention include its relative speed, cost, and the ability to rapidly test large numbers of compounds. Significantly, in contrast to simple growth inhibition screens, the targeted
5 screening of the invention seeks to restrict drug discovery to compounds that act by specific mechanisms. The assays have been carefully designed to target an essential bacterial process that is not found in mammalian cells. Mammalian cells do not regulate transcription or translation via the
10 ppGpp pathway (Silverman et al., 1979, Microbiol. Rev. 43:27-41). Therefore, compounds which affect the ppGpp pathway have the potential to be both very effective in controlling bacterial growth while causing less side effects to the infected host.

15 The methods of the present invention provide an efficient, focused approach to drug discovery with significant improvements over previous methods. One major improvement is a method for increasing the efficiency of drug discovery by ensuring that lead compounds are more likely to
20 affect their desired molecular target inside the test organism. Unlike the traditional approaches used for antimicrobial drug screening, which are either blind to the bacterial mechanism which is being targeted and/or do not provide for new classes of antibiotics, the present invention
25 targets a unique bacterial process. The screening methods described herein can identify compounds that cause ppGpp accumulation by *relA* independent mechanisms, such as, enhanced PSII activity or inhibition of ppGpp degradase. Using the present approach new classes of compounds may be
30 found, and efficacy and selectivity of these compounds can be optimized.

4. DEFINITIONS

35 ppGpp - either or both guanosine nucleotide analogs of GDP and GTP, bearing a pyrophosphate group esterified to the 3'-hydroxyl of the ribose moiety.

PSI - ppGpp synthase I which is encoded at the *relA* locus.

PSII - ppGpp synthase II which is not encoded at the *relA* locus.

5 *relA*⁺ independent pathway(s) - biochemical pathway(s) of ppGpp

synthesis or degradation not catalyzed by protein(s) encoded at the *relA* locus.

AT - 3-amino-1,2,4-triazole

10 *in vitro* - occurring outside a whole cell, such as in an reaction using purified components.

in vivo - occurring within a living organism, such as an assay using growth of whole bacterial cells.

15 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for identifying compounds that are capable of causing the accumulation of ppGpp in bacteria.

In one embodiment, the invention provides methods
20 for the identification of compounds that enhance the activity of ppGpp synthase II (PSII). In another embodiment, the invention provides methods for the identification of compounds that inhibit the activity of ppGpp degradase. The methods comprise screening assays in which test compounds are
25 brought into contact with test cells, and observing the effect such compounds have on ppGpp levels in the test cells.

In general, the methods of the invention involve contacting a test compound with *relA* deficient test cells for a time sufficient to allow the test compound to cause ppGpp
30 accumulation, determining the level of ppGpp or the effect of ppGpp accumulation, and comparing the level or effect obtained to the level or effect in a test cell which has not been contacted with the test compound, such that if the level or effect of ppGpp in the cells contacted with the test
35 compound is different from those cells not contacted, the test compound is identified as a potential candidate.

In a less preferred embodiment, *relA*⁺ strains of test cells can also be used in the methods of the invention.

Instead of measuring directly the level of ppGpp in the test cells, the invention provides two strategies for
5 detecting the accumulation of ppGpp efficiently. The first strategy is based on detecting paradoxical growth of test cells under stress conditions; such growth is affected by ppGpp level which regulates certain biosynthetic pathways. The other strategy employs a reporter gene the expression of
10 which is under the control of a promoter that is either negatively or positively regulated by ppGpp. Methods based on these strategies can readily be automated and adapted for high throughput screening.

In another embodiment, the invention provides
15 genetically manipulated *relA*⁻ test cells which contain a reporter gene the expression of which is sensitive to the level of ppGpp. Both reporter mRNAs and reporter molecules in test cells can by design be easily detected and quantitated by techniques known in the art.

20 The term "test cell" as used herein referred to bacterial cells that are generally deficient at the *relA* locus or its equivalent (*relA*⁻), and are preferably *spoT*⁺. Generally, the test cells have been genetically manipulated for use in the screening assays of the invention. The term
25 also encompasses any progeny of the subject test cell.

The term "ppGpp" is used in this invention to designate collectively either or both ppGpp (guanosine tetraphosphate) or pppGpp (guanosine pentaphosphate). The nucleotide pppGpp is converted to ppGpp *in vivo* by removal of
30 the 5' terminal phosphate by the product of the *gpp*⁻ gene (pppGpp 5'-phosphohydrolase), and, most likely, to a lesser extent by the product of the *ppx* gene (exopolyphosphatase) (Koonin, 1994, Trends Biochem. Sci., 19:156-157; Kuroda et al. 1997, J. Biol. Chem. 272:21240-21243). The product of
35 the *spoT* gene can degrade pppGpp or ppGpp to GTP or GDP, respectively, by removal of the 3'pyrophosphate. For the purposes of this invention, pppGpp and ppGpp are essentially

interchangeable in their function as regulatory molecules, since accumulation of the former leads rapidly to accumulation of the latter.

The term "ppGpp accumulation" as used herein, 5 refers to the accumulation of ppGpp or pppGpp inside test cells of the invention in which the synthesis or degradation is not catalysed by protein(s) encoded at the *relA* locus. Generally, ppGpp accumulation in test cells of the invention results from the activity of ppGpp synthase II (not ppGpp 10 synthase I), and/or the relative lack of activity of ppGpp degradase. The activities of PSII and ppGpp degradase in test cells are targets in the screening assays of the invention.

The term "test compound" refers to a compound to be 15 tested by one or more screening assays of the invention as a putative agent that causes ppGpp accumulation. The test compounds of the invention encompass numerous classes of chemical molecules, though typically they are organic molecules, and preferentially of low molecular weight. 20 Typically, these compounds have a molecular weight of more than about 50, but less than about 3,000, and preferably less than 1,000.

Test compounds are obtained from a wide variety of sources including collections of natural products in the form 25 of bacterial, fungal, plant and animal extracts; and synthetical chemical libraries. Numerous means known in the art are available for the random, directed and combinatorial synthesis of a wide variety of chemical structures. In addition, natural products or known antibiotic compounds may 30 be subjected to random or directed chemical modifications to produce derivatives and structural analogs for use as test compounds in the invention. Usually various predetermined concentrations are used for screening such as 0.001 μM , 0.01 μM , 0.1 μM , 1.0 μM , 10 μM , and 100 μM .

35 Test compounds that score positive in the screening assays of the invention are putative agents that cause ppGpp accumulation, and are useful as leads for the development of

therapeutic agents useful for the treatment of infectious diseases. Drugs based on such agents are likely to be both very effective in controlling growth in a broad spectrum of bacteria while causing minimal side effects to the treated
5 subject.

The invention further provides biochemical assays which are used to further study the test compounds which produced positive results in the screening assays of the invention.

10 For clarity of discussion, the invention is described in the subsections below by way of example of *E. coli*. However, the principles may be applied to other bacteria which use similar mechanisms for regulation of intracellular ppGpp level.

15

5.1. REGULATION OF ppGpp

While not limited to any theory on how the level of ppGpp is regulated in bacteria, the methods of the invention are based in part on our understanding of the mechanisms
20 involving ppGpp through which bacteria adapts to changes in the environment.

Bacteria generally exist in rapidly changing nutritional environments. To optimize growth and division in these various environments, bacteria developed sophisticated
25 gene control mechanisms to allow the organism to produce particular enzymes in quantities that is optimal for the environment. Similarly, the translation apparatus itself is controlled by growth conditions.

For purposes of the treatment of human diseases
30 caused by bacteria, understanding these gene control mechanisms provides an opportunity to look for compounds which adversely affect the bacterial genes that control growth. With this in mind, applicants have developed a strategy for screening for potential antibiotic drugs by
35 identifying compounds which cause an accumulation of ppGpp via a *relA* independent pathway such as, for example, enhancing PSII activity or inhibiting ppGpp degradase. The

assays of the invention provide a highly sensitive system that can be used to detect compounds that would otherwise be overlooked by ppGpp assays which have been used to study gene function in bacteria.

- 5 The genes encoding ribosomal RNA ("rRNA") respond rapidly to changes in the environment or in the growth medium. A reduction of rRNA synthesis in response to amino acid starvation is known as the "stringent response." During amino acid starvation, it is advantageous to down-regulate
10 rRNA synthesis since the need for ribosomes is reduced when one of their substrates, i.e., amino acids, is in limited supply.

- ppGpp appears to be responsible for negative regulation of rRNA synthesis during the stringent response.
15 In *E. coli*, the PSI enzyme encoded at the *relA* is activated during amino acid starvation. The PSI enzyme is located on the ribosome and is activated by codon-specific binding of uncharged tRNA in the ribosomal RNA acceptor site (Haseltine and Block, 1973, Proc. Natl. Acad. Sci. USA 70:1564-1568;
20 Cochran and Byrne, 1974, J. Biol. Chem. 249:353-360; Pedersen and Kjeldgaard, 1977, Eur. J. Biochem 76:91-97).

- During amino acid starvation, it is advantageous to down-regulate rRNA synthesis since the need for ribosomes is reduced when one of their substrates, i.e., amino acids, is
25 in limited supply. In contrast, synthesis of amino acids must be up-regulated to overcome the starvation condition. This regulatory scheme assures that cellular energy is not wasted on *de novo* biosynthesis when amino acids are available in the growth medium, or on ribosome synthesis when amino
30 acid supplies are exhausted.

- Although the stringent response was first characterized as a response to amino acid starvation, it is now recognized that ppGpp levels change in response to a variety of other stress conditions, including carbon,
35 nitrogen or phosphate starvation, heat shock, osmotic shock and pH changes (Murray and Bremer, 1996, J. Mol. Biol. 259:41-57; Cashel et al., 1996, *Escherichia coli* and

Salmonella Cellular and Molecular Biology, ed. Neidhardt, F.C. ASM Press, Washington, D.C. pp. 1458-1496). Although the stringent response was first described in *E. coli*, it is known to be a general stress response and ppGpp has been
5 found in all eubacteria tested (M. Cashel, 1994, Methods in Molecular Genetics, vol. 3, Molecular Microbiology Techniques, Part A, ed. Adolph, K.W. (Academic Press, New York) pp. 341-356)).

The *relA* structural gene consists of 743 codons
10 encoding a protein of about 84 kDa. The *relA* gene product has two domains, one for ribosomal binding and the other for ppGpp synthetic activity (Schreiber et al., 1991, J. Biol. Chem. 266:3760). The *relA* gene or its equivalent in other bacteria is expected to share a high degree of sequence
15 homology with the *relA* gene of *E. coli*. Such a gene in other bacteria may be identified by techniques commonly known in the art, such as hybridization assays using the *relA* gene as a probe.

RelA mutants, or relaxed mutants, are well
20 characterized. A large number of relaxed mutants with RNA accumulation behavior ranging from slightly to strongly relaxed were isolated and characterized by Fiil and Friesen, 1968, J. Mol. Biol. 45:195-203; Fiil and Friesen, 1969, J. Bacteriol. 95:729-731. Relaxed mutants have increased
25 permeability to glutamate (Broda, 1968, *Escherichia coli*. J. Bacteriol. 96:1528-1534) and increased sensitivity to numerous inhibitors (Pao and Gallant, 1978, Mol. Gen. Genet. 158:271-277; Stephens et al., 1975, Proc. Natl. Acad. Sci. USA 72:4389-4393).

30 Two plate growth tests, both based on positive regulatory effects of ppGpp accumulation on amino acid biosynthesis, have been used to study *relA* mutants. The first test, involves mutant sensitivity to glucose minimal medium supplemented with serine, methionine, and glycine (SMG)
35 (Donachie, 1968, Nature 219:1077-1079); the other test, known as the AT plate test, involves sensitivity to 3-amino-1,2,4-

triazole (AT) which is an inhibitor of histidine biosynthesis (Rudd et al., 1985, J. Bacteriol. 163:534-542).

PSII (ppGpp synthetase II), encoded by the *SpoT* gene, is the second known enzyme which catalyzes the
5 synthesis of ppGpp. PSII is one of the drug target in the present invention. PSII is not activated by amino acid starvation but is active during exponential growth and during certain conditions of environmental stress e.g., phosphate source starvation (Hernandez and Bremer, 1993, J. Biol. Chem.
10 268:10851-10862). Levels of ppGpp during exponential growth are determined by PSII activity. *Id.*

Relatively less is known about the products of the *SpoT* gene. The *E. coli* PSII enzyme has not been characterized biochemically and attempts to obtain homogenous
15 protein from *E. coli* have been unsuccessful suggesting that the enzyme is functionally or physically unstable (Richter, 1969, In Ribosomes (Chambliss, Gaven, Davis, Davis, Kahan and Nomura eds.) 743-765, Univ. Park Press, Baltimore, MD.). PSII activity has, however, been found in certain *Bacillus*
20 organisms (Sy and Akers, 1976, Biochemistry 15:4399-4403; Fehr and Richter, 1981, J. Bacteriol. 145:68-73). PSII activity is found in the cytoplasm. *ibid.* *SpoT* (ppGpp degradase) is the major enzyme activity causing turnover of ppGpp.

25 It had been suggested that the *SpoT* gene encodes a single product which may be a bifunctional enzyme capable of catalyzing both ppGpp synthesis and degradation, or that the *SpoT* gene could encode a regulation of both degradation or synthesis of ppGpp (Cashel and Rudd, 1987; Metzger et al.,
30 1989, J. Biol. Chem. 264:9122-9125; Hernández and Bremer, 1991, J. Biol. Chem. 266(9):5991-5999; Xiao et al., 1991, J. Biol. Chem. 266(9):5980-5990). PSII activity is reported to be generated during or shortly after *spoT* mRNA translation. Regulation of *spoT* encoded activities appears to be
35 responsible for the growth medium-dependent changes in basal levels of ppGpp. PSII appears to catalyze ppGpp synthesis in a ribosome independent fashion. Although the identity of the

PSII enzyme has not been proven, the evidence that *spoT* is the structural gene for PSII and is not simply asserting a regulatory effect is based on the fact that ppGpp is not detectable in strains with deletions in both *relA* and *spoT* (Xiao et al., 1991, J. Biol. Chem. 266(9):5980-5990) and the fact that *RelA* and *SpoT* have extensive amino acid sequence homology throughout their length. Metzger et al., 1989 J. Biol. Chem. 264:9122-9125.

Further, analysis of the *E. coli spoT* gene has recently identified distinct but overlapping regions involved in ppGpp synthesis and degradation (Gentry and Cashel, 1996, Molecular Microbiol. 19(6):1373-1384). The region containing the first 203 amino acids of the 702 amino acid *SpoT* protein was confirmed to have ppGpp degradase activity. An overlapping region containing residues 67-374 conferred PSII activity. *Id.* In addition, PSII activity of *B. stearothermophilus* appears to reside in a monomeric enzyme whose molecular mass is about the same as that of the *E. coli SpoT* product. Fehr and Richter, 1981 J. Bacteriol. 145:68-73.

The effects of ppGpp are pleiotropic, and metabolism of ppGpp is in fact central to many vital bacterial processes. Cellular functions affected by ppGpp include nucleotide metabolism, amino acid metabolism, heat shock and cold shock proteins, basic DNA binding proteins, transport, carbohydrate metabolism, cell wall synthesis and others (Cashel et al., 1996, *Escherichia coli* and *Salmonella* Cellular and Molecular Biology, ed. Neidhardt, F.C. ASM Press, Washington, D.C., pp. 1458-1496). ppGpp binds directly to the β -subunit of RNA polymerase (Reddy et al., 1995, Mol. Microbiol. 15:255-265). The ppGpp-RNA polymerase complex has a reduced affinity for rRNA and tRNA promoters, so that ribosomal synthesis is decreased when ppGpp accumulates (Zhang & Bremer, 1995, J. Biol. Chem. 370:11181-11189).

The metabolic cycle of ppGpp, as described below, involves the rapid conversion of the nucleotide pppGpp to

ppGpp *in vivo* by removal of the 5' terminal phosphate by the product of the *gppA* gene (pppGpp 3'-phosphohydrolase) and possibly by the product of the *ppx* gene. The term ppGpp is therefore used in this invention to designate collectively
5 either or both of these nucleotides, which are essentially interchangeable in their function as regulatory molecules. ppGpp is degraded by ppGpp degradase which is a specific Mn⁺⁺-requiring 3'-pyrophosphohydrolase encoded by the *SpoT* gene. The cycle is closed through the action of nucleoside
10 5'-diphosphate kinase (*nkd* product) on GDP to give GTP (Metzger et al., 1989 J. Biol. Chem. 264:9122-9125). Degradation of ppGpp by the *SpoT* protein is reduced during carbon source downshift. Net degradation of ppGpp has been found to occur in numerous bacterial systems (Richter et al.,
15 1979, Eur. J. Biochem. 99:57-64).

Despite extensive sequence similarity, *SpoT* does not share the ribosomal association that has been attributed to *RelA* (Gentry & Cashel, 1995, J. Bacteriol. 177:3890-3893). Although physiologically less active, degradation of ppGpp
20 has been reported to occur via less active *SpoT* independent routes. For example, weak activity has been found in cell extracts (An et al., 1979, J. Bacteriol. 137:1100-1110; Heinemeyer et al., 1978, Eur. J. Biochem. 89:125-131). It is contemplated that such *SpoT*-independent ppGpp degradase
25 activities are also drug targets of the invention for identifying compounds that cause ppGpp accumulation.

Another model of rRNA synthesis control, known as the ribosome feedback regulation model, assumes a repressor role of free or translating ribosomes, either directly or via
30 their activity, without involving a control of PSII activity and ppGpp synthesis (Jinks-Robertson and Nomura, 1987, in *Escherichia coli* and *Salmonella* Cellular and Molecular Biology, ed. Neidhart, F.C. (ASM Press, Washington, D.C.) pp. 1358-1385; Cole et al., 1987 J.Mol. Biol. 198:383-392).
35 However, later reports have questioned this result.

Further, it has also been suggested, using ppGpp-less strains without detectable ppGpp synthase activity

(ppGpp^o) that ppGpp is not required for the growth rate control of rRNA synthesis (Gaal and Gourse, 1990, Proc. Natl. Acad. Sci. USA 87:5533-5537). However, Hernandez and Bremer, 1993, J. Biol. Chem. 25:10851-10862, found that growth rate-
5 dependent adjustments of the total RNA synthesis rate do require ppGpp.

5.2. SCREENING ASSAYS OF THE INVENTION

The methods of the invention are designed to
10 identify compounds that cause ppGpp accumulation in test cells. Generally, the screening assays comprise contacting a test compound with test cells for a time sufficient to allow the test compound to cause ppGpp accumulation, and
15 determining the level of ppGpp or the effect of ppGpp accumulation.

The contacting may be effected in any vehicle and by any means using standard protocols, such as serial dilution, and the use of wells, or disks impregnated with a solution or suspension of a test compound. The amount of
20 time allowed for the test compound to cause ppGpp accumulation in the test cells may be determined empirically, such as by running a time course and monitoring the effect of ppGpp accumulation as a function of time. For example, the test cells may be grown in culture to reach a certain phase
25 or density, and then the test cells are exposed to a test compound; or alternatively, the test cells may be grown continuously in the presence of a test compound.

In one embodiment, the invention provides an assay that is based on a *relA*⁻, *spoT*⁺ strain of test cells which
30 grows normally in rich medium, but not under certain stress conditions as ppGpp synthesis from PSII will be inadequate for normal growth. Compounds that cause ppGpp accumulation by enhancing PSII activity or inhibiting ppGpp degradase allow "paradoxical growth" of the test cells under the stress
35 condition since the test cell would not normally be able to grow. Such compounds are detected by plating the test cells on specialized growth medium that creates these stress

conditions. Stress conditions that may be used include but are not limited to metabolic stress, such as carbon or nitrogen starvation, osmotic shock, and pH changes. Examples of such media, not by way of limitation, include minimal medium containing serine hydroxamate, or minimal medium containing the histidine antimetabolite, 3-amino-1,2,4-triazole (AT). Both of these media induce an amino acid starvation condition. Table I shows expected growth of the assay strain in rich medium or on starvation-inducing medium such as mentioned above, in the presence or absence of a ppGpp degradase inhibitor at an appropriate concentration. Typically the appropriate concentration is identified by testing many concentrations as occur, for example, when the compound is impregnated in a paper disk which is then placed on an agar-based medium.

TABLE I

Medium	Effect of test compound on ppGpp levels	Growth
Rich	Increase	(+)
Rich	Decrease	(+)
Starvation-inducing	No effect	(-)
Starvation-inducing	Increase	(+)
Starvation-inducing	Decrease	(-)

Accordingly, the invention provides a method for screening for test compounds that cause ppGpp accumulation in bacteria comprising:

- (a) contacting a *relA* deficient test cell under a stress condition such as those mentioned above with a test compound for a time sufficient to allow the test compound to cause ppGpp accumulation in the test cell; and

- (b) detecting growth of the test cell under the stress condition, wherein an increase in growth of the test cell contacted with the test compound relative to the growth of the test cell not contacted with the test compound, indicates that the test compound causes ppGpp accumulation in the test cell.

In another embodiment, the invention provides a screening assay that uses a strain of *E. coli* with reduced ppGpp synthetic activity and normal degradase activity (*relA*, *spoT*⁺), but also carrying a reporter gene, such as *lacZ* (β -galactosidase). This reporter gene is under the control of a promoter that is either negatively or positively controlled by ppGpp.

In one specific embodiment, the expression of the reporter gene in test cells is placed under the control of a promoter that is negatively regulated by ppGpp. By negative regulation is meant that the promoter is transcriptionally active in the absence of ppGpp, and is transcriptionally inactive in the presence of ppGpp. That is, the reporter gene product is produced only when ppGpp levels are low. Under normal conditions, the test cells produce some ppGpp by the PSII enzyme, but this ppGpp is degraded by the degradase activity, and the overall ppGpp levels are low. When the ppGpp level is low, the reporter gene is expressed. In the presence of a compound which, for example, enhances PSII activity or inhibits ppGpp degradase, ppGpp accumulates and as a result, transcription of the reporter gene is repressed. The lack of expression or downregulation of expression of the reporter gene indicates a rise in ppGpp level in the test cells, and identifies the test compound as a candidate for further studies.

In a preferred embodiment, the reporter gene is under the control of a promoter of a ribosomal RNA-encoding gene that is negatively controlled by ppGpp. The test cells are grown on rich medium. For example, and not by way of limitation, the *E. coli* strain VH2733 may be used. This strain contains a *relA* deletion. Further, the strain

contains a *lacZ* reporter gene operably linked to the *rrnB* P1 promoter. The *rrnB* P1 promoter is one of the promoters of the rRNA genes (*rrn*) and is negatively controlled by ppGpp. The *rrnB* P1 promoter is initiated by RNA polymerase. When
5 ppGpp is present, it attaches to the RNA polymerase, modifying its structure and activity. This complex cannot form a proper initiation complex at the *rrnB* P1 promoter. Thus, transcription of *rrnB* P1 will be limited in the presence of ppGpp. In this strain, the only source of β -
10 galactosidase (β -Gal) is the *rrnB* P1-linked *lacZ* gene. The genotype and construction of the VH2733 strain is described in Hernandez, 1991, J. Biol. Chem. 266(9):5991-5999 and Hernandez and Bremer, 1990, J. Biol. Chem. 265(20):11605-11614, both of which are incorporated by reference in their
15 entirety for all purposes.

In a highly preferred embodiment, the assay uses a strain designated VH2736 as deposited with the American Type Culture Collection (ATCC), Rockville, Maryland, on February 10, 1998, and assigned accession number _____. This *relA*
20 mutant also contains the *spoT*⁺ wildtype, and also contains the *lacZ* gene fused to the *rrnB* P1 promoter but contained in a more robust bacterial strain, MG1655. The relevant genotype of VH2736 is MG1655 (*E. coli* K12 wildtype) Δ *relA* 251::KAN *spoT*⁺ *malB*::P1-*lacZ*::KAN. The strain VH2736 was
25 made from MG1655 by introducing the Δ *relA* 251::KAN (described in Xiao et al., 1991, J. Biol. Chem. 266 (9):5980-5990) and *malB*::P1-*lacZ*::KAN (described in Hernandez and Bremer, 1990, J. Biol. Chem. 265 (20): 11605-11614) genes by techniques well known to those skilled in the
30 art. Both references are incorporated herein in their entirety.

The preferred assay strains accumulate a low level of ppGpp from an equilibrium of the PSII enzyme and ppGpp degradase activities. Under these conditions, the *lacZ*
35 reporter gene is expressed and the assay strain is effectively *lac*⁺. In the presence of a compound which, for example, enhances PSII activity or causes ppGpp degradase

inhibition, ppGpp accumulates to high levels and therefore transcription of *lacZ* is inhibited, and the assay strain becomes effectively *lac*⁻.

Activity of the *lacZ* protein can be detected by any
5 medium that allows differentiation of *lac*⁻ from *lac*⁺ bacterial growth (e.g., lactose and 2,3,5-triphenyl-2H-tetrazolium chloride [tetrazolium], 5-bromo-4-chloro-3-indolyl- β -D-galactoside [X-gal], MacConkey's, or other any such medium known in the art). The preferred medium is lactose-
10 tetrazolium indicator agar. On this medium, *lac*⁺ growth is white and *lac*⁻ growth is red. The 2,3,5-triphenyl-2H-tetrazolium chloride present in the medium will be reduced by any cell growth to form an insoluble formazan red dye. However, if the cells can metabolize lactose and reduce the
15 pH in the surrounding medium, formation of the red dye is prevented. In the absence of a desired compound such as one, for example, which is a ppGpp degradase inhibitor, the strain accumulative low levels of ppGpp such that the overall levels are too low to inhibit *lacZ* expression. Therefore, *LacZ* gene
20 product is produced and bacterial growth is white. In the presence of a compound that inhibits ppGpp degradase, ppGpp accumulates to high levels, the *lacZ* gene is and bacterial growth is red. Eventually, if ppGpp levels are driven high enough, they will become toxic and limit growth. Therefore,
25 desired compounds will also cause a zone of growth inhibition around the point of application. In the ideal case, desired compounds will cause a zone of growth inhibition with red growth at the periphery. Past this periphery cells should grow normally and be white. Table 2 shows expected results
30 when the assay strain is grown in lactose-tetrazolium medium, in the presence or absence of a compound which causes ppGpp accumulation.

TABLE II

5	Effect of test compound on ppGpp levels	ppGpp levels	lacZ transcription	Color of growth
	No effect	Baseline	(+)	White
	Increase	High	(-)	Red
	Decrease	Low	(+)	White

10 Accordingly, the invention provides a method for screening for test compounds that cause ppGpp accumulation in bacteria comprising:

- 15 (a) contacting a *relA* deficient test cell with a test compound for a time sufficient to allow the test compound to cause ppGpp accumulation in the test cell, wherein the test cell contains a reporter gene operably linked to a promoter which is negatively regulated by ppGpp, and
- 20 (b) detecting the expression of the reporter gene in the test cell, wherein a decrease in the expression of the reporter gene in the test cell contacted with the test compound relative to the expression of the reporter gene in a test cell not contacted with the test compound, indicates that the test
- 25 compound causes ppGpp accumulation in the test cell.

In another specific embodiment, the expression of the reporter gene in test cells is placed under the control of a promoter that is positively regulated by ppGpp. By

30 positive regulation is meant that the promoter requires ppGpp for full transcriptional activity. That is, the reporter gene product is produced only when ppGpp levels are high. In the test cells, transcription of the reporter gene is up-regulated when ppGpp levels are high.

35

In a preferred embodiment, the assay uses a strain of *E. coli* with reduced PSI activity and normal degradase

activity as described above (*relA*⁻, *spoT*⁺), with the reporter gene under the control of a promoter that is positively controlled by ppGpp. For example, and not by way of limitation, the reporter gene, such as but not limited to the
5 β -galactosidase (*lacZ*) gene, may be placed under the control of a promoter from a gene encoding a protein involved in histidine biosynthesis, such as *hisP*₁, *hisP*₂. Previous studies indicated that *spoT* mutants with defective ppGpp degradation activity show overexpression of the *his* operon
10 due to elevated ppGpp levels (Rudd et al., 1985, J. Bacteriol. 163:534-542 (in *Salmonella typhimurium*); and Sarubbi et al., 1988, Mol. Gen. Genet. 213:214-222 (in *Escherichia coli*).)

The test cells are plated on rich medium on which
15 the test cells grow and produce some ppGpp by the PSII enzyme. But this ppGpp is degraded by the degradase activity and the overall ppGpp levels are low. Under these conditions, there is only a low background level of reporter gene expression from the positively regulated promoter, since
20 ppGpp is required for full expression. In the presence of a compound which causes the accumulation of ppGpp to high levels, such as, for example, a ppGpp degradase inhibitor, ppGpp accumulates and therefore the reporter gene is fully expressed.

25 Activity of the reporter gene product, such as β -galactosidase, can be detected by any medium that allows differentiation of *lac*⁻ from *lac*⁺ bacterial growth (e.g., lactose-tetrazolium, 5-bromo-4-chloro-3-indolyl- β -D-galactoside [X-gal], MacConkey's, or the like). The
30 preferred medium contains X-gal. On this medium, β -galactosidase activity results in a blue color. In the absence of such a ppGpp degradase inhibitor, negligible β -galactosidase is produced and the bacterial growth is only slightly blue. In the presence of a compound that inhibits
35 ppGpp degradase, ppGpp accumulates and allows maximum β -galactosidase production giving the bacterial growth a pronounced blue color which is easily distinguishable from

the much lighter blue background. Again, desired compounds will cause a zone of growth inhibition with a dark blue ring at the periphery. Table 3 shows the expected results when the test cells are grown in a X-gal-containing medium, in the presence or absence of a compound which causes ppGpp accumulation.

TABLE III

10	Effect of test compound on ppGpp levels	ppGpp levels	lacZ transcription	Color of growth
15	No effect Increase Decrease	Baseline High Low	Baseline (+) (-)	White Blue White

Accordingly, the invention provides a method for screening for test compounds that cause ppGpp accumulation in bacteria comprising:

- 25 (a) contacting a *relA* deficient test cell with a test compound for a time sufficient to allow the test compound to cause ppGpp accumulation in the test cell, wherein the test cell contains a reporter gene operably linked to a promoter which is positively regulated by ppGpp, and
- 35 (b) detecting the expression of the reporter gene in the test cell, wherein an increase in the expression of the reporter in the test cell contacted with the test compound relative to the expression of the reporter gene in a test cell not contacted with the test compound, indicates that the test compound causes ppGpp accumulation in the test cell.

It should be noted that, since *in vitro* and *in vivo* conditions provide different environments, certain compounds

which give a certain result *in vitro* result will not necessarily give the same result *in vivo*. For example, two compounds that have been reported to inhibit ppGpp degradase *in vitro* did not test positive in the assay of the invention:
5 tetracycline and picolinic acid. Both have been reported to indirectly inhibit by chelation of manganese (manganese is required for ppGpp degradase activity).

In the case of tetracycline, inhibition was reported in an *in vitro* reaction when the antibiotic was
10 added to a final concentration of about 400 $\mu\text{g/ml}$ (Richter, 1980, Arch. Microbiol. 124:229-232). This concentration would be impossible to achieve in whole cells since concentrations above 50 $\mu\text{g/ml}$ completely inhibit bacterial growth. Thus, the lack of a red ring is explained by the
15 discrepancy between the concentration needed for degradase inhibition and the concentration that can be tolerated by growing bacteria.

Picolinic acid is a hydrophobic compound which is only sparingly soluble in water, so diffusion through the
20 solid agar and away from the point of application is inefficient. In fact, picolinate did not cause a zone of inhibition in the agar assay although it inhibited growth in liquid cultures.

25 5.3. TEST CELLS OF THE INVENTION

The test cells of the invention are employed in screening assays for the identification of compounds that cause ppGpp accumulation.

In one embodiment, the invention uses a strain of
30 test bacterial cells that is deficient at the *relA* locus or its equivalent. Such strains (also referred to as "relaxed" strains) are known in the art. These cells lack PSI activity which synthesizes ppGpp in quantities sufficient to inhibit ribosomal RNA synthesis under certain physiological
35 conditions. The *relA* locus or its equivalent in these cells may either be deleted or mutated to the extent that the gene product is no longer functional. To make a *relA* bacterial

strain, the *relA* gene or its equivalent in wildtype bacteria may be mutagenized or deleted by genetic methods or recombinant DNA techniques well known in the art. These cells may also be *spoT*⁺ which provides normal PSII activity
5 and normal ppGpp degradase activity.

In another embodiment, the invention uses a strain of test bacterial cells that is deficient in *relA* or its equivalent, and that comprises a genetic sequence encoding a reporter molecule, wherein the expression of said genetic
10 sequence is regulated by positively or negatively by ppGpp.

In a more specific embodiment, the *relA* test cells comprise a reporter construct comprising a genetic sequence encoding a reporter molecule (i.e., a reporter gene sequence), said genetic sequence is operably linked to a
15 ppGpp-responsive promoter, i.e., a promoter which is regulated positively or negatively by the level of ppGpp in the test cells.

In various embodiments, the reporter gene sequence of the invention can include any genetic sequence, preferably
20 DNA sequence which encodes a detectable gene product (i.e., a peptide or polypeptide). The genetic sequences encoding detectable reporter gene products are well known to those of skill in the art. The reporter gene sequence does not encode necessarily a detectable peptide or polypeptide, since the
25 messenger RNAs of the reporter gene sequence can be detected and quantified.

Any *relA* bacterial cells that can express a reporter gene under the control of a ppGpp-responsive promoter may be used. Test bacterial cells may be obtained
30 from private laboratory deposits, public culture collections such as the American Type Culture Collection, or from commercial suppliers. It is desirable to use bacteria which have been developed for drug screening processes, and that conditions for their growth, maintenance, and manipulations
35 are known.

The most preferred bacterial species that is useful as test cells is *Escherichia coli*. Other preferred bacterial

species may include but not limited to *Bacillus subtilis*, and *Pseudomonas aeruginosa*. For example, and not by way of limitation, a highly preferred strain of test cells is *E. Coli* strain VH2736, as deposited with the American Type Culture Collection (ATCC), Rockville, Maryland, on February 10, 1998.

Escherichia coli can be used as a model of many bacteria. Test compounds that specifically inhibit host transcription of *E. coli* test cells are expected to have a similar inhibitory effect on the transcription of pathogenic species, such as but not limited to, *Vibrio* species, *Pseudomonas* species, *Acinetobacter* species, *Bordetella* species, *Campylobacter* species, *Haemophilus* species, *Neisseria* species and Enterobacteriaceae species, such as *Salmonella*, *Enterobacter*, *Klebsiella*, *Yersinia*, *Proteus*, *Serratia*, and *Staphylococcus* species, *Streptococcus* species, *Corynebacterium* species, *Listeria* species and *Bacillus* species. It is also expected that positive test compounds will be effective as an antibiotic against multidrug-resistant strains of these pathogenic species, such as β -lactam-resistant strains of *E. coli*.

Standard molecular biology techniques can be used to construct reporter gene constructs containing the ppGpp-responsive promoter and translational control signals if necessary, reporter gene sequence, and other regulatory sequence, such as terminators.

In the present invention, the reporter gene sequence(s) may be inserted into a recombinant expression vector. The term "reporter gene constructs" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of reporter gene sequences. Such reporter gene constructs of the invention are preferably plasmids which contain a ppGpp-responsive promoter sequence which is operably associated with the inserted reporter gene sequence. It typically contains an origin of replication as well as specific genes which allow phenotypic selection of the transformed cells.

"Operably-associated" or "operably-linked" refers to an association in which the heterologous promoter and the reporter gene sequence are joined and positioned in such a way as to permit transcription. Two or more sequences, such as a promoter and any other nucleic acid sequences are operably-associated if transcription commencing in the heterologous promoter will produce an RNA transcript of the operably-associated sequences.

A reporter gene construct useful in the invention may also contain selectable or screenable marker genes for initially isolating, identifying or tracking test cells that contain heterologous DNA. The reporter gene construct may also provide unique or conveniently located restriction sites to allow severing and/or rearranging portions of the DNA inserts in a reporter gene construct. More than one reporter genes may be inserted into the construct such that the test cells containing the resulting construct can be assayed by different means.

Introduction of the reporter gene construct into bacterial cells DNA may be carried out by conventional techniques well known to those skilled in the art, such as transformation, conjugation, and transduction. For example, where the host is *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth and subsequently treated by the CaCl_2 method using procedures well known in the art. Alternatively, MgCl_2 or RbCl could be used.

In addition to conventional chemical methods of transformation, the plasmid vectors of the invention may be introduced into a host cell by physical means, such as by electroporation or microinjection. Electroporation allows transfer of the vector by high voltage electric impulse, which creates pores in the plasma membrane of the host and is performed according to methods well known in the art. Additionally, cloned DNA can be introduced into host cells by protoplast fusion, using methods well known in the art.

The host cells which contain the reporter gene sequence and which express the reporter gene product may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of
5 "marker" gene functions (e.g., resistance to antibiotics); (c) assessing the level of transcription as measured by the expression of reporter mRNA transcripts in the host cell; and (d) detection of the reporter gene product as measured by immunoassay or by its biological activity.

10 The test cells may be cultured under standard conditions of temperature, incubation time, optical density, plating density and media composition corresponding to the nutritional and physiological requirements of the bacteria. However, conditions for maintenance and growth of the test
15 cell may be different from those for assaying candidate test compounds in the screening methods of the invention. Modified culture conditions and media are used to facilitate detection of the expression of a reporter molecule. Any techniques known in the art may be applied to establish the
20 optimal conditions.

Test cell strains, cell cultures, cell lines generated by the above-described methods for the screening assays may be expanded, stored and retrieved by any techniques known in the art that is appropriate to the test
25 cell. For example, the test cells of the invention can be preserved by stab culture, plate culture, or in glycerol suspensions and cryopreserved in a freezer (at -20°C to -100°C) or under liquid nitrogen (-176°C to -196°C).

The term "reporter gene" as used herein refers to
30 any genetic sequence that is detectable and distinguishable from other genetic sequences present in test cells. Preferably, the reporter gene sequence encodes a protein that is readily detectable either by its presence, or by its activity that results in the generation of a detectable
35 signal. A reporter gene is used in the invention to monitor and report the activity of a ppGpp-responsive promoter in test cells.

A reporter gene encodes a reporter molecule which is capable of directly or indirectly generating a detectable signal. Generally, although not necessarily, the reporter gene encodes RNA and detectable protein that are not

5 otherwise produced by the test cells. Many reporter genes have been described, and some are commercially available for the study of gene regulation. See, for example, Alam and Cook, 1990, Anal. Biochem. 188:245-254, the disclosure of which is incorporated herein by reference.

10 Any antigenic peptide or protein that can be detected by an antibody can be used as a reporter, for example, growth hormone (Selden et al., Mol. Cell Biol., 6:3173). To facilitate detection by antibody binding in immunoassays, antigenic reporter molecules that are secreted
15 or attached on the test cell surface are preferred.

For convenience and efficiency, enzymatic reporters and light-emitting reporters are preferred for the screening assays of the invention. Accordingly, the invention encompasses histochemical, colorimetric and fluorometric
20 assays.

A variety of enzymes may be used as a reporter which includes but are not limited to β -galactosidase (Nolan et al. 1988, Proc. Natl. Acad. Sci. USA 85:2603-2607), chloramphenicol acetyltransferase (CAT; Gorman et al., 1982,
25 Mol Cell Biol, 2:1044; Prost et al., 1986, Gene 45:107-111), β -lactamase, β -glucuronidase and alkaline phosphatase (Berger et al., 1988, Gene 66:1-10; Cullen et al., 1992, Methods Enzymol; 216:362-368). Transcription of the reporter gene leads to production of the enzyme in test cells. The amount
30 of enzyme present can be measured via its enzymatic action on a substrate resulting in the formation of a detectable reaction product. The methods of the invention provides means for determining the amount of reaction product, wherein the amount of reaction product generated or the remaining
35 amount of substrate is related to the amount of enzyme activity. For some enzymes, such as β -galactosidase, β -glucuronidase and β -lactamase, fluorogenic substrates are

available that allow the enzyme to covert such substrates into detectable fluorescent products (see, for example, U.S. Patent No. 5,070,012, and WO 96/30540).

The most preferred reporter gene of the invention
5 is the *LacZ* gene encoding *E. coli* β -galactosidase. The enzyme is very stable and has a broad specificity so as to allow the use of different chromogenic or fluorogenic substrates, such as but not limited to lactose 2,3,5-triphenyl-2H-tetrazolium (lactose-tetrazolium), 5-bromo-4-
10 chloro-3-indolyl- β -D-galactoside (X-gal), and fluorescein galactopyranoside (Molecular Probes, Orgeon). See, Nolan et al. 1988, Proc. Natl. Acad. Sci. USA 85:2603-2607.

Another commonly used reporter gene is the *E. coli* β -glucuronidase gene (GUS; Gallagher, 1992, in "GUS
15 protocols", Academic Press) which can be used with various histochemical and fluorogenic substrates, such as X-glucuronide, and 4-methylumbelliferyl glucuronide.

A variety of bioluminescent, chemiluminescent and fluorescent proteins can also be used as light-emitting
20 reporters in the invention. One type of such reporters, which are enzymes and require cofactor(s) to emit light, include but are not limited to, the bacterial luciferase (*luxAB* gene product) of *Vibrio harveyi* (Karp, 1989, Biochim. Biophys. Acta 1007:84-90; Stewart et al. 1992, J Gen
25 Microbiol, 138:1289-1300), and the luciferase from firefly, *Photinus pyralis* (De Wet et al. 1987, Mol. Cell. Biol. 7:725-737).

Another type of light-emitting reporter, which does not require substrates or cofactors, includes but are not
30 limited to the wild type green fluorescent protein (GFP) of *Victoria aequoria* (Chalfie et al. 1994, Science 263:802-805), and modified GFPs (Heim et al. 1995, Nature 373:663-4; PCT publication WO 96/23810). Transcription and translation of this type of reporter gene leads to the accumulation of the
35 fluorescent protein in test cells, which can be measured by a device, such as a fluorimeter, or a flow cytometer. Methods for performing assays on fluorescent materials are well known

in the art and are described in, e.g., Lackowicz, J.R., 1983, Principles of Fluorescence Spectroscopy, New York:Plenum Press.

Depending on the screening technique and nature of the signal used to assay the reporter gene expression, a reporter regimen can be used to aid directly or indirectly the generation of a detectable signal by a reporter molecule. A reporter regimen comprises compositions that enable and support signal generation by the reporter, such as substrates and cofactors for reporter molecules that are enzymes; e.g., lactose-tetrazolium medium. Such compositions are well known in the art. Components of a reporter regimen may be supplied to the test cells during any step of the screening assay.

5.4. SPECIFIC EMBODIMENTS

The invention may be better understood by the following description of illustrative embodiments which are not intended to be limiting.

5.4.1. DETERMINATION OF PARADOXICAL GROWTH

This embodiment uses an *E. coli* strain such as, and not by way of limitation, VH2733 or VH2736, with normal PSII activity and normal degradase activity (*spoT*⁺), but lacking PSI activity due to a mutation or deletion at the *relA* locus.

The assay strain is plated as a lawn on solid medium containing AT, for example, at a concentration of 15mM. Test compounds are then applied to the medium in wells or on disks. Paradoxical growth is determined by visually comparing growth around the well or disk containing the test compound to growth in control areas which are free of the test compound. Comparison of test and control areas is done at the same time point. Compounds that cause ppGpp accumulation show a ring of enhanced growth at the periphery of a zone of growth or inhibition around the point of application. Compounds which cause ppGpp accumulation, such as NaN₃ (sodium azide) (Murray and Bremer, 1996, J. Mol. Biol. 259:41-57), or 1,10-phenanthroline are included in the assay

as a control since the test strain is not expected to grow on the medium in the absence of a ppGpp degradase inhibitor. That is, paradoxical growth is seen around the points of application, and this growth diminishes as NaN_3 concentration decreases.

5.4.2. **DETERMINATION OF ppGpp ACCUMULATION
USING A REPORTER GENE NEGATIVELY
CONTROLLED BY ppGpp**

In this embodiment, the assay strain is an *E. coli* strain which is a *relA* mutant with reduced PSI synthetase activity but with normal or wildtype PSII synthetase activity and degradase activity (*spoT*⁺). This strain also contains the reporter gene *lacZ* under the control of a promoter which is negatively controlled by ppGpp. The strain is plated as a lawn on solid lactose tetrazolium medium (50 $\mu\text{g/ml}$ 2,3,5-triphenyl-2H tetrazolium chloride), and compounds are added in wells, as described in Section 5.4.1. The cells added to the agar plate are actively growing (log phase) but dilute enough to avoid crowding of cells and inaccurate results. Colony color around the well or disk containing test compound is compared to control areas of the plate that are free of test compound. Comparison of test and control areas is done at the same time point. Compounds that cause ppGpp accumulation are expected to show a ring of red growth around the well or disk. In other lactose differential medium, results will be analogous but the positive and negative readout will depend on the indicator of lactose fermentation. Compounds that cause ppGpp accumulation, such as NaN_3 , 1,10-phenanthroline or picolinic acid is included in the assay as a positive control. At high concentrations NaN_3 (sodium azide) is toxic, and therefore there is a zone of inhibited bacterial growth close to the disk. At some distance from the disk, a ring of red growth is seen, indicating that at some lower concentration NaN_3 is allowing ppGpp accumulation and the cells have become *lac*⁻.

Accordingly, the invention provides a method for screening for test compounds that cause ppGpp accumulation in bacteria comprising:

- 5 (a) culturing a strain of *relA* deficient test cell which contains a β -galactosidase gene operably linked to a promoter that is negatively regulated by ppGpp, such as *E. coli* strain VH2736;
- 10 (b) inoculating a plate containing a medium comprising lactose and 2,3,5-triphenyl-2H-tetrazolium with the test cells;
- (c) adding a test compound to a point of application, such as a well, in the plate and incubating the plate for a time sufficient to allow the test compound to contact the test cell and cause ppGpp accumulation;
- 15 (d) detecting a red coloration and growth of the test cells contacted with the test compound, wherein the red coloration indicates a lack of expression of the β -galactosidase gene in the test cells; and
- 20 (e) comparing the coloration and growth of test cells not contacted with the test compound;

wherein an increase in red coloration and growth of the test cell contacting the test compound relative to the coloration and growth of test cells not contacting the test compound indicates that the test compound causes ppGpp accumulation in the test cell.

5.4.3. DETERMINATION OF ppGpp ACCUMULATION USING A REPORTER GENE POSITIVELY 30 CONTROLLED BY ppGpp

In this embodiment, the assay strain is a *relA* deficient mutant with reduced PSI synthetase activity but with normal PSII synthetase activity and normal degradase activity with a *lacZ* reporter gene under the control of a promoter which is positively controlled by ppGpp. This strain is plated as a lawn on solid medium, and test compounds are added in wells, as described in Section 5.4.1.

The medium can be LB (as described in Maniatis et al., MOLECULAR CLONING A Laboratory Manual, Cold Spring Harbor Press, eds. 1982) or similar rich medium containing X-gal (80 $\mu\text{g/ml}$) as a chromogenic substrate for the β -galactosidase enzyme. Colony color around the well or disk containing test compound is compared to control areas of the plate that are free of test compound. Comparison of test and control areas is done at the same time point. Compounds that cause ppGpp accumulation are expected to show a ring of blue growth around the well or disk. In other lactose differential medium, results will be analogous but the positive and negative readout will depend on the indicator of lactose fermentation. A known inhibitor of ppGpp degradase, such as NaN_3 , 1,10-phenanthroline or picolinic acid is included in every plate as a positive control. At high concentrations this compound is toxic, and therefore there is a zone of inhibited bacterial growth close to the disk. At some distance from the disk, a ring of blue growth is seen, indicating that at some lower concentration, NaN_3 is allowing ppGpp accumulation and the cells have become lac^+ .

Accordingly, the invention provides a method for screening for test compounds that cause ppGpp accumulation in bacteria comprising:

- (a) culturing a *relA* deficient strain of *E. coli* test cell wherein the test cells contains a β -galactosidase gene operably linked to a promoter which is positively regulated by ppGpp;
- (b) inoculating a plate containing a medium comprising 5-bromo-4-chloro-3-indolyl- β -D-galactoside with the test cells;
- (c) adding a test compound to a point of application, such as a well, in the plate and incubating the plate for a time sufficient to allow the test compound to contact the test cell and cause ppGpp accumulation;
- (d) detecting a blue coloration and an inhibition of growth of the test cells contacted with the test

compound, wherein the blue coloration indicates expression of the β -galactosidase gene in the test cells; and

- (e) comparing the coloration and growth of test cells not contacted with the test compound;
wherein an increase in blue coloration, and an inhibition of growth of the test cell contacting the test compound relative to the coloration and growth of test cells not contacting the test compound indicates that the test compound causes ppGpp accumulation in the test cell.

5.5. ELIMINATION OF β -GALACTOSIDASE INHIBITORS

Compounds that inhibit β -galactosidase enzymatic activity will give false positive results in the primary assay of Section 5.4.2. Such compounds can be eliminated by testing against a standard preparation of β -galactosidase protein using a commercial kit with a chemiluminescent substrate (Tropix), or by repeating the primary assay using a strain of bacteria that expresses *lacZ* from a promoter that is not responsive to ppGpp. Compounds that inhibit β -galactosidase are considered false positive and are not carried forward. All compounds which do not inhibit β -galactosidase are potential inhibitors of ppGpp degradase and are carried through to the secondary assays described below.

Alternatively, after screening by using the methods described in the primary assay in Section 5.4.2., elimination of false positive results may be carried out by using a second screening such as, for example, the methods described in Section 5.4.3.

5.6. TLC ANALYSIS OF ppGpp

Intracellular levels of ppGpp can be accurately detected and quantitated by labeling cells *in vivo* with ^{32}P orthophosphate, which is incorporated into all phosphorylated cellular components including ATP, GTP, pppGpp and ppGpp. The labeled nucleotide products are extracted using formic

acid, then separated (TLC) by thin layer chromatography. The addition of putative ppGpp degradase inhibitors to cultures of cells being labeled should lead to an increase in intracellular ppGpp. Thus, a TLC assay will establish
5 promising compounds as true modulators of intracellular ppGpp levels.

Methods for TLC known in the art may be used such as the TLC assay (M. Cashel, 1994, Methods in Molecular Genetics, vol. 3, Molecular Microbiology Techniques, Part A,
10 ed. Adolph, K.W. (Academic Press, New York) pp. 341-356) which is incorporated by reference in its entirety. Accumulation of ppGpp over basal levels is detected in cells grown in liquid media with defined phosphate concentrations (approximately 0.4 mM) low enough to achieve ³²P specific
15 activities enabling nucleotide detection. Other methods of labeling well known in the art are also applicable. Cells are uniformly labeled by growth in the presence of radioactive orthophosphate (200 - 250 μ Ci/mM) for two generations prior to the addition of test compound. Labeled
20 nucleotides are extracted by adding concentrated formic acid and freeze-thawing the cell suspension. Labeled nucleotides are resolved by TLC, using polyethyleneimine cellulose (PEI) plates in a concentrated phosphate buffer solvent. This solvent lowers background by continuously eluting excessive
25 radioactive phosphate present in extracts. The separated nucleotide species are visualized by autoradiography. Quantitation can be done by cutting out radioactive spots and counting them in a liquid scintillation counter, or by densitometry or other methods known to those skilled in the
30 art. A diluted aliquot of an unchromatographed sample can be used as a baseline for calculating the phosphate specific activity achieved in the cell and, in turn, the relative abundance of ppGpp, ppGpp and GTP.

5.7. DETERMINATION OF ENZYMATIC ACTIVITY USING PARTIALLY PURIFIED ppGpp DEGRADASE

Active ppGpp degradase (SpoT protein) can be isolated from *E. coli* and used in an in vitro enzyme assay in order to assess the effects of compounds on the partially purified enzyme.

5.7.1. PREPARATION OF RADIOLABELED SUBSTRATE

Ribosomes with active ppGpp synthetase I (*relA*) are prepared from a strain of *E. coli* that carries a plasmid encoding the *relA* gene under the control of an inducible promoter. These ribosomes are then combined with GTP and ATP, and the *relA* protein synthesizes both ppGpp and pppGpp. CF3120 cells are grown in Luria broth containing 100 µg/ml ampicillin to an A_{600} of 1.5. *relA* expression is then induced by the addition of IPTG to a final concentration of 1 mM. Cells are incubated for 1 hour, then harvested by centrifugation. The cell pellet is washed in ribosomal buffer (50 mM Tris acetate [pH 8.0], 15 mM Mg acetate, 60 mM potassium acetate, 27 mM ammonium acetate, 1 mM DTT and 0.2 mM EDTA) and the resulting cell pellet is stored at -70°C. The frozen cell pellet is resuspended in 2 volumes (w/v) of ribosomal buffer then cells are lysed by French press. The lysate is centrifuged at 11,000 x g for 40 min at 4°C. The supernatant is centrifuged at 30,000 rpm in a Beckman Ti65 (or equivalent) for 4 hrs at 4°C. The resulting pellet of ribosomes and membranes is combined with 2.5 volumes of cold ribosomal buffer, transferred to a beaker and stirred slowly overnight at 4°C. The solution is then centrifuged at 7,500 x g for 15 min at 4°C to remove undissolved debris. The supernatant is removed and ribosomal buffer is added to bring the suspension to 4X (w/v) with respect to the original weight of the cells. A 5 ml cushion of 40% sucrose in ribosomal buffer is placed in a 30 ml ultracentrifuge tube then the ribosomal suspension is carefully layered on top, filling the tube. The preparation is centrifuged at 32,000 rpm in a Beckman Ti65 (or equivalent) for 4 hrs at 4°C. The

supernatant is discarded and the pellet is transferred to a beaker containing a minimal volume of cold ribosomal buffer. The mixture is stirred at 4°C until resuspended then stored by dropping drops into a beaker filled with liquid nitrogen. 5 The drops freeze and can be stored in vials at -70 °C. Just before use, drops are transferred to a tube and thawed on ice.

The ribosomes are used to synthesize radiolabeled ppGpp and pppGpp as follows. A reaction mixture containing 10 0.5 mM GTP (mixed with ³²P alpha GTP), 2 mM ATP and ribosome preparation is incubated overnight at room temperature. The reaction is stopped by adding phenol-chloroform, and diluted to 5 ml with 20 mM Tris-Cl (pH 6.8), 6 M urea, 0.1 mM EDTA and 40 mM LiCl. Both ppGpp and pppGpp are produced in the 15 reaction.

To separate the reaction components (ppGpp, pppGpp, GTP and ATP), the reaction mixture is loaded to a 0.9 x 40 cm QAE-Sephadex column equilibrated in the same buffer. The column is washed in the same buffer, then the reaction 20 products are eluted with a linear gradient of LiCl from 0.1 mM to 0.5 mM. The separation of ppGpp, pppGpp, GTP and ATP is assessed by TLC and autoradiography, then separate pools are made of fractions containing ppGpp or pppGpp. Either of these products can be used in the degradase reaction 25 described below. The pooled fractions are diluted 10-20 fold in 20 mM Tris-Cl (pH 6.8), 0.1 mM EDTA and 40 mM LiCl, then applied to a 1 ml QAE-Sephadex column equilibrated in the same buffer. The ppGpp or pppGpp is eluted with 4 M LiCl, then precipitated by adding 5 volumes of ethanol (at -20°C). 30 The mixture is centrifuged and the pellet is resuspended in TE and stored at -20°C.

5.7.2. PREPARATION OF ACTIVE ppGpp DEGRADASE

Active ppGpp degradase is prepared from a strain of 35 *E. coli* that overexpresses *spoT*. In a preferred embodiment, overexpression of *spoT* is accomplished by induction of an IPTG-responsive promoter in a plasmid carrying the gene for

spoT. Cells are grown at 30°C in Luria broth supplemented with 0.2% glucose, 40 mM potassium phosphate (pH 7.5) and appropriate antibiotics. When the culture reaches an A600 of 1.0, expression of *spoT* is induced by the addition of IPTG to a final concentration of 1 mM. Cells are incubated for an additional 2 hrs, then harvested by centrifugation, washed in lysis buffer (50 mM Tris acetate, [pH 8.0], 5 mM EDTA, 0.23 M NaCl, 100 µg/ml PMSF, 1 mM DTT), then stored at -80°C. The frozen cell pellet is resuspended in 3 volumes of lysis buffer and cells are lysed using a French press. The resulting cell lysate is centrifuged at 11,000 x g. *SpoT* protein is found in both the supernatant and the pellet. *SpoT* protein is extracted from the pellet by adding 50 ml TGED buffer containing 1M NaCl and stirring at 4°C for 1 hr (TGED buffer = 10 mM Tris, [pH 8.0], 5% glycerol, 0.1 mM EDTA, 0.1, 0.1 mM DTT). Ammonium sulfate is added to the extracted pellet solution and to the supernatant, to a final volume of 25%. The mixture is stirred at 4°C for 1 hr, then precipitated proteins (including *SpoT*) are recovered by centrifugation at 11,000 x g. The pellet is dissolved in 7 ml of TGED buffer containing 1M NaCl and 50% glycerol, then applied to a 200 ml gel filtration column (for example, Sephacryl 200) equilibrated with TGED containing 1M NaCl. Fractions containing *SpoT* protein are pooled and dialyzed against *SpoT* storage buffer (TGED buffer containing 1M NaCl and 10% glycerol). The resulting *SpoT* preparation is stored at -70 °C. The preparation contains partially purified active *SpoT* protein and can be used for enzyme assays. If further purification is desired, the preparation is applied to a 5 ml heparin column (for example, BioRad) that has been equilibrated with 300 mM NaCl, 5% glycerol. *SpoT* protein is eluted with a 100 ml continuous gradient of 300 mM to 1M NaCl, 5% glycerol. Fractions containing *SpoT* are pooled and stored at -70 °C in *SpoT* storage buffer.

35

5.7.3. DEGRADASE ASSAY

Purified SpoT protein is combined with radiolabeled pppGpp (or ppGpp) 10X reaction buffer (500 mM Tris-Cl (pH 8.0), 1M Mg acetate, 10 mM DTT) and MnCl₂ to a final concentration of 1 mM. The reactions are incubated, with and without added test compound, at room temperature. Aliquots are removed at various time points up to 20 minutes (for example, 0.5, 1, 2, 5, 10 and 20 min) and spotted to PEI TLC plates. The reaction substrate and products are separated by developing the TLC plate in phosphate buffer and visualized by autoradiography. The amount of product (GTP and pyrophosphate) can be quantitated by liquid scintillation counting or other methods known in the art. Degradase activity can be compared in the presence and absence of test compound, thus verifying the effects of *bona fide* inhibitors of degradase enzymatic activity.

5.8. DETERMINATION OF MIC

The minimum inhibitory concentration (MIC) against bacterial organisms is determined for each compound that is positive in both the primary assay (using lactose tetrazolium as described in Section 5.4.2) and secondary assay (using X-gal as described in Section 5.4.3). Methods known in the art may be used such as broth microdilution testing, using a range of concentrations of each test compound (1993, National Committee for Clinical Laboratory Standards). Methods for Dilution Antimicrobial Susceptibility Tests For Bacteria That Grow Aerobically - Third Edition: Approved Standard, M7-A3). The MIC against a variety of pathogens are determined using the same method. Pathogenic species to be tested generally include: *E. coli*, *Enterococcus faecium*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus epidermis*, *Shigella flexneri*, and *Salmonella typhimurium*.

35

5.9. CYTOTOXICITY TESTING

Unfortunately, toxicity does not always arise from the same mechanism of action as is responsible for growth inhibition in the targeted microorganism. Therefore, the
5 selectivity of the target should not be assessed solely on the basis of these results.

Cytotoxicity can be measured by methods known in the art. One such method is assessing growth of mammalian cells in the presence of the test compound, using a protein binding
10 dye, sulforhodamine B (SRB). SRB binds electrostatically to basic amino acids. Binding and solubilization of the dye can be controlled by changes in pH. SRB binds stoichiometrically to proteins in one pH range but can be solubilized and extracted for measurement in another. An increase in total
15 protein is correlated to cell growth. Cell growth in the presence of compound is compared to growth without added compound to establish a growth inhibitory concentration (GI₅₀) (Skehan et al., 1990, J. Natl. Cancer. Inst., 82:1107-1112). Another method of measuring cytotoxicity which may be used in
20 an assay containing 3[4,5-dimethylthiazol-2-yl]-2,5,-diphenyltetrazolium bromide/2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt ("MTT/XTT") as described in Mosmann T., 1983, J. Immunol. Methods, 65:55-63, which is incorporated by reference in its
25 entirety for all purposes.

5.10. ANTIBIOTIC AGENTS IDENTIFIED BY METHODS OF THE INVENTION

In yet another embodiment, the invention provides
30 novel antibiotic agents discovered by the methods described above. These antibiotic agents are capable of causing ppGpp accumulation in a bacterial cell, leading to downregulation of rRNA synthesis, and ultimately to a reduction or inhibition of bacterial growth. These agents may, for
35 example, act by enhancing PSII activity, and/or inhibiting ppGpp degradase activity, and are expected to be effective in a variety of species of bacteria, including infectious

pathogenic bacteria. The invention also includes novel pharmaceutical compositions which comprise antibiotic agents discovered as described above formulated in pharmaceutically acceptable formulations.

5 In another embodiment, the invention features a method for treating a subject infected with an infectious agent by administering to that subject a therapeutically effective amount of an antibiotic agent which causes ppGpp accumulation (for example, by enhancing PSII activity, and/or
10 inhibiting ppGpp degradase activity) in the infectious agent as determined by the assays of the invention. Such administration can be by any method known to those skilled in the art, for example, by topical application or by systemic administration.

15 In yet another embodiment, antibiotic agents of the present invention can be used to treat contaminated items, such as crops, wood, metal or plastic and the like, by methods such as, but not limited to, spraying or dusting of that agent onto the contaminated item, or impregnating that
20 agent into the item.

By "therapeutically effective amount" is meant an amount that relieves (to some extent) one or more symptoms of the disease or condition in the patient. Additionally, by "therapeutically effective amount" is meant an amount that
25 returns to normal, either partially or completely, physiological or biochemical parameters associated with or causative of a bacterial disease or condition.

5.10.1. Formulation

30 The antibiotic compounds identified by methods of the invention may be formulated into pharmaceutical preparations for administration to animals for treatment of a variety of infectious diseases. Compositions comprising a compound of the invention formulated in a compatible
35 pharmaceutical carrier may be prepared, packaged, labelled for treatment of and used for the treatment of the indicated

infectious diseases caused by microorganisms, such as those listed infra in Section 5.9.3.

- If the antibiotic compound is water-soluble, then it may be formulated in an appropriate buffer, for example, 5 phosphate buffered saline or other physiologically compatible solutions. Alternatively, if the resulting complex has poor solubility in aqueous solvents, then it may be formulated with a non-ionic surfactant such as Tween, polyethylene glycol or glycerine. Thus, the compounds and their 10 physiologically acceptable solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral, topical, dermal, vaginal, rectal administration and drug delivery device, e.g., porous or viscous material, such as lipof foam.
- 15 For oral administration, the pharmaceutical preparation may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by 20 conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, or fractionated vegetable oils); and 25 preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize 30 starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents 35 (e.g., sodium lauryl sulphate). The tablets may be coated by methods well-known in the art.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration, the compositions may
5 take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from
10 pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a
15 valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral
20 administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or
25 emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

30 The antibiotic compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously,
35 the antibiotic compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or

intramuscularly) or by intramuscular injection. Thus, for example, the antibiotic compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or
5 as sparingly soluble derivatives, for example, as a sparingly soluble salt. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophilic drugs.

The antibiotic compositions may, if desired, be
10 presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

15 The pharmaceutical compositions of the present invention comprise an antibiotic compound as the active ingredient, or a pharmaceutically acceptable salt thereof, and may also contain a pharmaceutically acceptable carrier, and optionally, other therapeutic ingredients, for example
20 antivirals. The term "pharmaceutically acceptable salts" refers to salts prepared from pharmaceutically acceptable non-toxic acids and bases, including inorganic and organic acids and bases.

The pharmaceutical compositions include
25 compositions suitable for oral, rectal, mucosal routes, transdermal, parenteral (including subcutaneous, intramuscular, intrathecal and intravenous), although the most suitable route in any given case will depend on the nature and severity of the condition being treated.

30 In practical use, an antibiotic agent can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation
35 desired for administration, e.g., oral or parenteral (including tablets, capsules, powders, intravenous injections or infusions). In preparing the compositions for oral dosage

form any of the usual pharmaceutical media may be employed, e.g., water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents, and the like; in the case of oral liquid preparations, e.g., suspensions, solutions, 5 elixirs, liposomes and aerosols; starches, sugars, micro-crystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents, and the like in the case of oral solid preparations e.g., powders, capsules, and tablets. In preparing the compositions for parenteral 10 dosage form, such as intravenous injection or infusion, similar pharmaceutical media may be employed, e.g., water, glycols, oils, buffers, sugar, preservatives and the like know to those skilled in the art. Examples of such parenteral compositions include, but are not limited to 15 Dextrose 5%w/v, normal saline or other solutions.

5.10.2. ADMINISTRATION

For administration to subjects, antibiotic compounds discovered by using the assays of the invention are 20 formulated in pharmaceutically acceptable compositions. The compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These compositions can be utilized *in vivo*, ordinarily in a mammal, preferably in a human, or *in* 25 *vitro*. In employing them *in vivo*, the compositions can be administered to the mammal in a variety of ways, including parenterally, intravenously, subcutaneously, intramuscularly, colonially, rectally, vaginally, nasally, orally, transdermally, topically, ocularly, or intraperitoneally.

30 As will be readily apparent to one skilled in the art, the magnitude of a therapeutic dose of an antibiotic compound in the acute or chronic management of an infectious disease will vary with the severity of the condition to be treated, the particular composition employed, and the route 35 of administration. The dose, and perhaps dose frequency, will also vary according to the species of the animal, the age, body weight, condition and response of the individual

subject. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, will be within the ambit of one skilled in the art.

Desirable blood levels may be maintained by a
5 continuous infusion of an antibiotic compound as ascertained by plasma levels. It should be noted that the attending physician would know how to and when to terminate, interrupt or adjust therapy to lower dosage due to toxicity. Conversely, the attending physician would also know how to
10 and when to adjust treatment to higher levels if the clinical response is not adequate (precluding toxic side effects).

In selected cases, drug delivery vehicles may be employed for systemic or topical administration. They can be designated to serve as a slow release reservoir, or to
15 deliver their contents directly to the target cell. Such vehicles have been shown to also increase the circulation half-life of drugs which would otherwise be rapidly cleared from the blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are
20 liposomes, hydrogels, cyclodextrins, and bioadhesive microspheres. These vehicles have been developed for chemotherapeutic agents.

Topical administration of agents is advantageous when localized concentration at the site of administration
25 with minimal systemic adsorption is desired. This simplifies the delivery strategy of the agent to the disease site and reduces the extent of toxicological characterization. Furthermore, the amount of material to be administered is far less than that required for other administration routes.

30 Antibiotic agents may also be systemically administered. Systemic absorption refers to the accumulation of agents in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: oral, intravenous,
35 subcutaneous, intraperitoneal, intranasal, intrathecal and ocular. Each of these administration routes exposes the agent to an accessible target.

5.10.3. Target Infectious Agents

The antibiotic compounds identified by the methods of the invention can be used to treat infectious diseases in animals, including humans, companion animals (e.g., dogs and cats), livestock animals (e.g., sheep, cattle, goats, pigs, and horses), laboratory animals (e.g., mice, rats, and rabbits), and captive or wild animals.

Specifically, infectious diseases caused by bacteria including but not limited to, gram positive cocci, such as Staphylococci (e.g., *S. aureus*), Streptococci (e.g., *S. pneumoniae*, *S. pyogenes*, *S. faecalis*, *S. viridans*); gram positive bacilli, such as *Bacillus* (e.g., *B. anthracis*), *Corynebacterium* (e.g., *C. diphtheriae*), *Listeria* (e.g., *L. monocytogenes*); gram negative cocci, such as *Neisseria* (e.g., *N. gonorrhoeae*, *N. Meningitidis*); gram negative bacilli, such as *Haemophilus* (e.g. *H. influenzae*), *Pasteurella* (e.g., *P. multocida*), *Proteus* (e.g., *P. mirabilis*), *Salmonella* (e.g., *S. typhi murium*), *Shigella* species, *Escherichia* (e.g., *E. coli*), *Klebsiella* (e.g., *K. pneumoniae*), *Serratia* (e.g. *S. marcescens*), *Yersinia* (e.g., *Y. pestis*), *Providencia* species, *Enterobacter* species, *Bacteroides* (e.g., *fragilis*), *Acinetobacter* species, *Campylobacter* (e.g., *C. jejuni*), *Pseudomonas* (e.g., *P. aeruginosa*), *Bordetella* (e.g., *B. pertussis*), *Brucella* species, *Fracisella* (e.g., *F. tularensis*), *Clostridia* (e.g., *C. perfringens*), *Helicobacter* (e.g., *H. pylori*), *Vibrio* (e.g., *V. cholerae*), *Mycoplasma* (e.g., *M. pneumoniae*), *Legionella* (e.g., *L. pneumophila*), *Spirochetes* (e.g. *Treponema*, *Leptospira* and *Borrelia*), *Mycobacteria* (e.g., *M. tuberculosis*), *Nocardia* (e.g., *N. asteroides*), *Chlamydia* (e.g., *C. trachomatis*), and *Rickettsia* species, can be treated by antibiotic drugs discovered by the methods of the invention.

6. EXAMPLES6.1. SCREENING ASSAY USING AN *E. coli* STRAIN
WITH A REPORTER GENE NEGATIVELY
REGULATED BY ppGpp

5

6.1.1. INOCULATION OF LB (LURIA BROTH)
WITH *E. coli* STRAIN VH2736

LB (Luria Broth) prepared as described in Maniatis, et al., *supra*, Section 5.4.3, was inoculated with the *E. coli* strain VH2736 by removing *E. coli* VH2736 glycerol stock from
10 -70°C freezer, scraping off a loopful of frozen culture with a sterile inoculating loop, and streaking onto a LB agar plate (+50µg/ml kanamycin) that had been warmed to room temperature. The plate was incubated at 37°C for 18-24 hours. Five single, isolated colonies were selected from the
15 plate. 25 mL of LB (+50µg/ml of kanamycin) was then added with a sterile pipet to an autoclaved 125mL Erlenmeyer flask. Using a sterile loop, the selected colonies were transferred from the plate to the LB master flask. The flask was incubated at 37°C while shaking for 16-18 hrs. at 250 rpm.
20 The LB plate with culture is stored at 2-8°C.

6.1.2. DILUTION OF OVERNIGHT CULTURE

The Erlenmeyer flask was removed from the 37°C incubator and using LB broth as a reference, the optical
25 density (OD) of a 1:10 dilution of the culture (dilute with LB broth) was determined, using a spectrophotometer set at 460 nm wavelength light and a 1 cm path length. The turbidity of the culture at 16-18 hours should be around 4.500-5.000 OD units at 460 nm. When the OD was appropriate,
30 a 1:5000 dilution of the culture with 1X Minimal medium (as described in Maniatis et al., MOLECULAR CLONING A Laboratory Manual, Cold Spring Harbor Press, eds. 1982) containing 50µg/ml of kanamycin was made.

35

6.1.3. INOCULATION OF LACTOSE-TETRAZOLIUM PLATES

16 ml of the 1:5000 dilution was pipetted and added directly to the surface of a 10 x 10 inch plate containing

lactose-tetrazolium medium (as described in Silhavey et al., Experiments with Gene Fusions, Cold Spring Harbor Press, 1984, ppGpp. 268-9). The lactose-tetrazolium plate contained approximately 300 ml of medium in a square plaque tray
5 (Stratagene, Inc.). The medium contained 25.5 g BBL-Base, antibiotic medium 2 (Becton Dickinson Microbiology Systems) 50 mg 2,3,5-triphenyl-2H tetrazolium chloride and 50 ml 20% beta-lactose solution per liter. The plate was swirled slowly to cover the entire surface evenly with the diluted
10 culture. After the culture covered the agar plate surface, the plate was tipped diagonally so that the remaining liquid accumulated in one corner. Using a sterile pipet, the remaining liquid was removed from the plate. The plate was then placed, lid off, in a laminar flow hood to dry for 2
15 hours.

6.1.4. ADDITION OF COMPOUNDS

3 μ l of each test compound from four 96-well plates were added to one assay plate. The assay plates had been
20 previously stamped by a 96-pin stamp which created holes in the agar surface with an automated pipetting machine (Microlab 2200 Automated Pipetting System, Hamilton Co.). An additional row of wells were added in the center of the plate for dilutions of known positive and negative compounds. The
25 following controls were added:

positive controls: phenanthroline, 10 mg/ml
polymyxin E, 10 mg/ml

negative controls: spectinomycin, 10 mg/ml
tetracycline, 1 mg/ml

30 The cells were incubated at 37°C for 24 hours.

6.1.5. SELECTION OF POSITIVE COMPOUNDS

A positive result was indicated by a red ring of growth around the well.

35

6.2. **SCREENING FOR COMPOUNDS WHICH CAUSE
ppGpp ACCUMULATION VIA AN *E. COLI*
STRAIN POSITIVELY REGULATED BY ppGpp**

The test compounds which yielded a positive result was then "retested" using a strain of *E. coli hisP₁lacZ (relA⁻, spoT⁺)* which contains the reporter *lacZ* gene under the control of positive promotor, the *hisP₁* promoter.

6.2.1. **INOCULATION OF LB (LURIA BROTH)
WITH *E. coli hisP₁lacZ (relA⁻, spoT⁺)***

LB (Luria Broth) prepared as described in Maniatis, et al., *supra*, Section 5.4.3, was inoculated with the *E. coli* strain *hisP₁lacZ (relA⁻, spoT⁺)* by removing *E. coli hisP₁lacZ (relA⁻, spoT⁺)* glycerol stock from -70°C freezer, scraping off a loopful of frozen culture with a sterile inoculating loop, and streaking onto a LB agar plate that had been warmed to room temperature. The plate was incubated at 37°C for 18-24 hours. Five single, isolated colonies were selected from the plate. 25 mL of LB was then added with a sterile pipet to an autoclaved 150mL Erlenmeyer flask. 125μl of sterile 40% dextrose solution was also added to the flask. Using a sterile loop, the selected colonies were transferred from the plate to the LB flask. The flask was incubated at 37°C while shaking for 16-18 hrs. at 250 rpm. The LB master plate with culture is stored at 2-8°C.

6.2.2. **DILUTION OF OVERNIGHT CULTURE**

The Erlenmeyer flask was removed from 37°C incubator and using LB broth as a reference, the OD of a 1:10 dilution of the culture (dilute with LB broth) was checked. The turbidity of the culture at 16-18 hours should be around 4.500-5.000. When the OD was appropriate, a 1:5000 dilution of the culture with 1X Minimal media was made.

6.2.3. **INOCULATION OF X-GAL PLATES**

16 ml of the 1:5000 dilution was pipetted and added directly to the surface of the X-Gal plate (LB agar plate with 80 μg/ml X-gal). The x-gal plate contained

approximately 300 ml of medium in a square plaque tray (Stratagene, Inc.). The plate was swirled slowly to cover the entire surface evenly with the diluted culture. After the culture covered the agar plate surface, the plate was

5 tipped diagonally so that the remaining liquid accumulated in one corner. Using a sterile pipet, the remaining liquid was removed from the plate. The plate was then placed, lid off, in a laminar flow hood to dry for 1 hour.

10 6.2.4. ADDITION OF COMPOUNDS

3 μ l of each test compound from four 96-well plates which yielded a positive result in Section 6.1 were added to one assay plate. The assay plates had been previously stamped by a 96-pin stamp which created holes in the agar surface with an automated pipetting machine (Microlab 2200 Automated Pipetting System, Hamilton Co.). An additional row of wells were added in the center of the plate for dilutions of known positive and negative compounds. The following controls were added:

20 positive controls: phenanthroline, 10 mg/ml
 polymyxin E, 10 mg/ml
 negative controls: spectinomycin, 10 mg/ml
 tetracycline, 1 mg/ml

The cells were incubated at 37°C for 24 hours.

25

6.2.5. SELECTION OF POSITIVE COMPOUNDS

Like the positive controls, a positive result was indicated by a compound with a dark blue ring of growth surrounding a zone of growth inhibition. Negative controls had zones of inhibition but no dark rings of growth.

6.3. TLC ANALYSIS

After getting a positive result in the second assay described in Section 6.2. TLC analysis was performed as described in Section 5.6. The results indicated that both phenanthroline and the "positive" agent from the assay in Section 6.2 gave positive results. By positive results is

meant accumulation of ppGpp in excess of control reactions without added compound.

7. DEPOSIT OF MICROORGANISMS

5 The following microorganism was deposited with the American Type Culture Collection (ATCC), Rockville, Maryland, on February 10, 1998 and assigned the indicated accession number:

	<u>Microorganism</u>	<u>ATCC Accession No.</u>
10	<i>E. coli</i> , VH2736	

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those
15 described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their
20 entireties.

25

30

35

MICROORGANISMSOptional Sheet in connection with the microorganism referred to on page 57, lines 5-20 of the description ***A. IDENTIFICATION OF DEPOSIT ***

Further deposits are identified on an additional sheet *

Name of depositary institution *

American Type Culture Collection

Address of depositary institution (including postal code and country) *

12301 Parklawn Drive
Rockville, MD 20852
USDate of deposit * February 10, 1998 Accession Number * _____**B. ADDITIONAL INDICATIONS *** (leave blank if not applicable). This information is continued on a separate attached sheet**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *** (if the indications are not all designated States)**D. SEPARATE FURNISHING OF INDICATIONS *** (leave blank if not applicable)The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g.,
"Accession Number of Deposit")E. ☐ This sheet was received with the International application when filed (to be checked by the receiving Office)_____
(Authorized Officer)☐ The date of receipt (from the applicant) by the International Bureau *

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

WHAT IS CLAIMED:

1. A method for screening for test compounds that cause ppGpp accumulation in bacteria comprising:

- 5 (a) contacting a *relA* deficient test cell under a stress condition with a test compound for a time sufficient to allow the test compound to cause ppGpp accumulation in the test cell; and
- 10 (b) detecting growth of the test cell under the stress condition, wherein an increase in growth of the test cell contacted with the test compound relative to the growth of the test cell not contacted with the test compound, indicates that the test compound causes ppGpp accumulation in the test cell.

15

2. A method for screening for test compounds that cause ppGpp accumulation in bacteria comprising:

- 20 (a) contacting a *relA* deficient test cell with a test compound for a time sufficient to allow the test compound to cause ppGpp accumulation in the test cell, wherein the test cell contains a reporter gene operably linked to a promoter which is negatively regulated by ppGpp, and
- 25 (b) detecting the expression of the reporter gene in the test cell, wherein a decrease in the expression of the reporter gene in the test cell contacted with the test compound relative to the expression of the reporter gene in a test cell not contacted with the test compound, indicates that the test
- 30 compound causes ppGpp accumulation in the test cell.

3. A method for screening for test compounds that cause ppGpp accumulation in bacteria comprising:

- 35 (a) contacting a *relA* deficient test cell with a test compound for a time sufficient to allow the test compound to cause ppGpp accumulation in the test

cell, wherein the test cell contains a reporter gene operably linked to a promoter which is positively regulated by ppGpp, and

- 5 (b) detecting the expression of the reporter gene in the test cell, wherein an increase in the expression of the reporter in the test cell contacted with the test compound relative to the expression of the reporter gene in a test cell not contacted with the test compound, indicates that
- 10 the test compound causes ppGpp accumulation in the test cell.

4. The method of claim 1 wherein the test cell further comprises a wildtype *spoT*⁺ locus.

15 5. The method of claim 2 wherein the test cell further comprises a wildtype *spoT*⁺ locus.

6. The method of claim 3 wherein the test cell

20 further comprises a wildtype *spoT*⁺ locus.

7. The method of claim 4, 5, or 6 wherein the test compound causes ppGpp accumulation in the test cell by enhancing ppGpp synthase II activity, or by inhibiting ppGpp

25 degradase activity.

8. The method of claim 4 wherein the test cell is an *E. coli*.

30 9. The method of claim 5 wherein the test cell is an *E. coli*.

10. The method of claim 6 wherein the test cell is an *E. coli*.

35 11. The method of claim 1 or 8 wherein the stress condition is caused by amino acid starvation.

12. The method of claim 1 or 8 wherein the stress condition is caused by culturing the test cell in minimal media containing 3-amino-1,2,4-triazol.

5 13. The method of claim 1 or 8 wherein the stress condition is caused by culturing the test cell in minimal media containing serine hydroxymate.

14. The method of claim 2 or 9 wherein the
10 reporter gene is operably associated with a promotor of a ribosomal RNA-encoding gene.

15. The method of claim 14 wherein the reporter gene is operably associated with the rrnB P1 promotor.

15

16. The method of claim 15 wherein the reporter gene is the E. coli β -galactosidase gene.

17. The method of claim 15 wherein the test cell
20 is cultured in a rich media comprising (i) lactose and 2,3,5-triphenyl-2H-tetrazolium, or (ii) 5-bromo-4-chloro-3-indolyl- β -D-galactoside.

18. The method of claim 3 or 10 wherein the
25 reporter gene is operably associated with a promotor for histidine biosynthesis.

19. The method of claim 18 wherein the reporter gene is operably associated with the hisP1 promoter or hisP2
30 promoter.

20. The method of claim 18 wherein the reporter gene is the E. coli β -galactosidase gene.

35 21. The method of claim 18 wherein the test cell is cultured in a rich media comprising (i) lactose and 2,3,5-

triphenyl-2H-tetrazolium, or (ii) 5-bromo-4-chloro-3-indolyl- β -D-galactoside.

22. The method of claim 17 wherein the test cell
5 is *E. coli* strain VH2733 or *E. coli* strain VH2736.

23. A method for screening for test compounds that
cause ppGpp accumulation in bacteria comprising:

- (a) culturing *E. coli* strain VH2736 test cells;
- 10 (b) inoculating a plate containing a medium comprising
lactose and 2,3,5-triphenyl-2H-tetrazolium with the
test cells;
- (c) adding a test compound to a point of application in
the plate and incubating the plate for a time
15 sufficient to allow the test compound to contact
the test cell and cause ppGpp accumulation;
- (d) detecting a red coloration and growth of the test
cells contacted with the test compound, wherein the
red coloration indicates a lack of expression of
20 the β -galactosidase gene in the test cells; and
- (e) comparing the coloration and growth of test cells
not contacted with the test compound;

wherein an increase in red coloration and growth of the test
cell contacted with the test compound relative to the
25 coloration and growth of test cells not contacted with the
test compound indicates that the test compound causes ppGpp
accumulation in the test cell.

24. A method for screening for test compounds that
30 cause ppGpp accumulation in bacteria comprising:

- (a) culturing a *relA* deficient strain of *E. coli* test
cell wherein the test cells contains a β -
galactosidase gene operably linked to a promoter
which is positively regulated by ppGpp;
- 35 (b) inoculating a plate containing a medium comprising
5-bromo-4-chloro-3-indolyl- β -D-galactoside with the
test cells;

- (c) adding a test compound to a point of application in the plate and incubating the plate for a time sufficient to allow the test compound to contact the test cell and cause ppGpp accumulation;
- 5 (d) detecting a blue coloration and an inhibition of growth of the test cells contacted with the test compound, wherein the blue coloration indicates expression of the β -galactosidase gene in the test cells; and
- 10 (e) comparing the coloration and growth of test cells not contacted with the test compound;
- wherein an increase in blue coloration, and an inhibition of growth of the test cell contacted with the test compound relative to the coloration and growth of test cells not
- 15 contacted with the test compound indicates that the test compound causes ppGpp accumulation in the test cell.

25. A method for screening for test compounds that cause ppGpp accumulation in bacteria comprising:

- 20 (a) contacting a *relA*⁺ test cell with a test compound for a time sufficient to allow the test compound to cause ppGpp accumulation in the test cell, wherein the test cell contains a reporter gene operably linked to a promoter which is negatively regulated
- 25 by ppGpp, and
- (b) detecting the expression of the reporter gene in the test cell, wherein a decrease in the expression of the reporter gene in the test cell contacted
- 30 with the test compound relative to the expression of the reporter gene in a test cell not contacted with the test compound, indicates that the test compound causes ppGpp accumulation in the test cell.

- 35 26. A method for screening for test compounds that cause ppGpp accumulation in bacteria comprising:

(a) contacting a *relA*⁺ test cell with a test compound for a time sufficient to allow the test compound to cause ppGpp accumulation in the test cell, wherein the test cell contains a reporter gene operably linked to a promoter which is positively regulated by ppGpp, and

(b) detecting the expression of the reporter gene in the test cell, wherein an increase in the expression of the reporter in the test cell contacted with the test compound relative to the expression of the reporter gene in a test cell not contacted with the test compound, indicates that the test compound causes ppGpp accumulation in the test cell.

27. A compound that causes ppGpp accumulation in bacteria that is identified by the method of claim 1, 2, or 3.

28. A pharmaceutical composition comprising a compound that causes ppGpp accumulation in bacteria that is identified by the method of claim 1, 2, or 3.

29. A method for the treatment of an infectious disease in animal comprising administering a therapeutically effective amount of a compound that causes ppGpp accumulation in bacteria that is identified by the method of claim 1, 2, or 3.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02565

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/02, 1/06, 1/10

US CL :435/4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG DATABASES: MEDLINE, BIOSIS PREVIEWS, CA SEARCH, WORLD PATENT INDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MUNRO, P.M. et al. Influence of the RpoS (KatF) Sigma Factor on Maintenance of Viability and Culturability of <i>Escherichia coli</i> and <i>Salmonella typhimurium</i> in Seawater. Applied and Environmental Microbiology. May 1995, Vol. 61, No. 5. pages 1853-1858, see entire article.	1-26
A	BREMER, H. et al. Guanosine tetraphosphate as a global regulator of bacterial RNA synthesis: a model involving RNA polymerase pausing and queuing. May 1995, Vol. 1262, No. 1, pages 15-36, see entire article.	1-26

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

28 MAY 1998

Date of mailing of the international search report

18 JUN 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
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Authorized officer

JOHNNY F. RAILEY II, PH.D.

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02565

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-26

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02565

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-26, drawn to methods for screening test compounds that cause ppGpp accumulation in bacteria.

Group II, claim 27, drawn to compounds that cause ppGpp accumulation in bacteria.

Group III, claim 28, drawn to pharmaceutical compositions.

Group IV, claim 29, drawn to a method for the treatment of an infectious agent in an animal.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I relates to screening methods using whole bacterial cells treated with any test compound of interest, wherein these compounds embrace both those types that are positively screened and those types that are negatively screened by the method used. Group II is drawn to an indefinite number of compounds that only are positively screened and identified by the methods of Group I, and hence these compounds are not commensurate with those which are used in the methods of Group I. The compounds of Group III represent only that subset of compounds of Group II which are not only positively screened by the methods of Group I, but are demonstrated useful as pharmaceuticals for treating animals or humans. Thus, these compounds are not commensurate with the compounds of Group II. The method of Group IV is distinct from the method of Group I and involves the use of compounds identified as pharmaceuticals from Group III. PCT Rules 13.1 and 13.2 do not provide for multiple distinct products and methods within a single application.

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